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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 9/28, C11D 3/386

A1

(11) International Publication Number: **WO 99/23211**

(43) International Publication Date: 14 May 1999 (14.05.99)

(21) International Application Number: PCT/DK98/00471

(22) International Filing Date: 30 October 1998 (30.10.98)

(30) Priority Data:

1240/97	30 October 1997 (30.10.97)	DK
PA 1998 00936	14 July 1998 (14.07.98)	DK

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: α–AMYLASE MUTANTS

(57) Abstract

The invention relates to a variant of a parent Termamyl–like α–amylase, which exhibits an alteration in at least one of the following properties relative to said parent α–amylase: i) improved pH stability at a pH from 8 to 10.5; and/or ii) improved Ca^{2+} stability at pH 8 to 10.5, and/or iii) increased specific activity at temperatures from 10 to 60 °C.

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α-amylase mutants

FIELD OF THE INVENTION

The present invention relates to variants (mutants) of parent Termamyl-like α-amylases with higher activity at medium temperatures and/or high pH.

BACKGROUND OF THE INVENTION

α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) constitute a group of enzymes which catalyze hydrolysis of starch and other linear and branched 1,4-glucosidic oligo- and polysaccharides.

There is a very extensive body of patent and scientific literature relating to this industrially very important class of enzymes. A number of α-amylases such as Termamyl-like α-amylases variants are known from *e.g.* WO 90/11352, WO 95/10603, WO 95/26397, WO 96/23873 and WO 96/23874.

Among more recent disclosures relating to α-amylases, WO 96/23874 provides three-dimensional, X-ray crystal structural data for a Termamyl-like α-amylase which consists of the 300 N-terminal amino acid residues of the *B. amyloliquefaciens* α-amylase (BAN™) and amino acids 301-483 of the C-terminal end of the *B. licheniformis* α-amylase comprising the amino acid sequence (the latter being available commercially under the tradename Termamyl™), and which is thus closely related to the industrially important *Bacillus* α-amylases (which in the present context are embraced within the meaning of the term "Termamyl-like α-amylases", and which include, *inter alia*, the *B. licheniformis*, *B. amyloliquefaciens* (BAN™) and *B. stearothermophilus* (BSG™) α-amylases). WO 96/23874 further describes methodology for designing, on the basis of an analysis of the structure of a parent Termamyl-like α-amylase, variants of the parent Termamyl-like α-amylase which exhibit altered properties relative to the parent.

BRIEF DISCLOSURE OF THE INVENTION

The present invention relates to novel α-amylolytic variants (mutants) of a Termamyl-like α-amylase which exhibit improved wash performance (relative to the parent α-amaylase) at high pH and at a medium temperature.

The term "medium temperature" means in the context of the invention a temperature from 10°C to 60°C, preferably 20°C to 50°C, especially 30-40°C.

The term "high pH" means the alkaline pH which is today used for washing, more specifically from about pH 8 to 10.5.

In the context of the invention a "low temperature α-amylase" means an α-amylase which has a relative optimum activity in the temperature range from 0-30°C.

In the context of the invention a "medium temperature α-amylase" means an α-amylase which has an optimum activity in the temperature range from 30-60°C. For instance, SP690 and SP722 α-amylases, respectively, are "medium temperature α-amylases.

In the context of the invention a "high temperature α-amylase" is an α-amylase having the optimum activity in the temperature range from 60-110°C. For instance, Termamyl is a "high temperature α-amylase.

Alterations in properties which may be achieved in variants (mutants) of the invention are alterations in:

The stability of the Termamyl-like α-amylase at a pH from 8 to 10.5, and/or the Ca^{2+} stability at pH 8 to 10.5, and/or the specific activity at temperatures from 10 to 60°C, preferably 20-50°C, especially 30-40°C.

It should be noted that the relative temperature optimum is often dependent on the specific pH used. In other words the relative temperature optimum determined at, e.g. pH 8, may be substantially different from the relative temperature optimum determined at, e.g., pH 10.

The temperature's influence on the enzymatic activity

The dynamics in the active site and surroundings are dependent on the temperature and the amino acid composition and of strong importance for the relative temperature optimum of an enzyme. By comparing the dynamics of medium and high temperature α-amylases, regions of importance for the function of high temperature α-amylases at medium temperatures can be determined. The temperature activity profile of the SP722 α-amaylase (SEQ ID NO: 2) and the *B. licheniformis* α-amylase (available from Novo Nordisk as Termamyl®) (SEQ ID NO: 4) are shown in Figure 2.

The relative temperature optimum of SP722 in absolute activities is shown to be higher at medium range temperatures (30-60°C) than the homologous *B. licheniformis* α-amylase, which has an optimum activity around 60-100°C. The profiles are mainly dependent on the temperature stability and the dynamics of the active site residues and their surroundings. Further, the activity profiles are dependent on the pH used and the pKa of the active site residues.

In the first aspect the invention relates to a variant of a parent Termamyl-like α-amylase, which variant has α-amylase activity, said variant comprises one or more mutations corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 2:

T141, K142, F143, D144, F145, P146, G147, R148, G149,
Q174, R181, G182, D183, G184, K185, A186, W189, S193, N195,
H107, K108, G109,D166, W167, D168, Q169, S170, R171, Q172, F173,
F267, W268, K269, N270, D271, L272, G273, A274, L275, K311,
E346, K385, G456, N457, K458, P459, G460, T461, V462, T463.

A variant of the invention have one or more of the following substitutions or deletions:

T141A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
K142A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
F143A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
D144A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F145A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
P146A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G147A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;

R148A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G149A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
R181*,A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G182*,A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D183*,A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G184*,A,R,D,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
K185A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
A186D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W189A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
S193A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
H107A,D,R,N,C,E,Q,G,I,L,K,M,F,P,S,T,W,Y,V;
K108A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G109A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D166A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W167A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
D168A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q169A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S170A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
R171A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q172A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F173A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
Q174*,A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F267A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
W268A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
K269A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N270A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D271A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L272A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
G273A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
A274D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L275A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
K311A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
E346A,D,R,N,C,Q,G,H,I,K,L,M,F,P,S,T,W,Y,V;
K385A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G456A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
N457A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K458A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

P459A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G460A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
T461A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
V462A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
T463A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V.

Preferred are variants having one or more of the following substitutions or deletions:
K142R; S193P; N195F; K269R,Q; N270Y,R,D; K311R; E346Q; K385R; K458R; P459T; T461P; Q174*; R181Q,N,S; G182T,S,N; D183*; G184*; K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V;A186T,S,N,I,V,R; 189T,S,N,Q.

Especially preferred are variants having a deletion in positions D183 and G184 and further one or more of the following substitutions or deletions:
K142R; S193P; N195F; K269R,Q; N270Y,R,D; K311R; E346Q; K385R; K458R; P459T; T461P; Q174*; R181Q,N,S; G182T,S,N; K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V; A186T,S,N,I,V,R; W189T,S,N,Q.

The variants of the invention mentioned above exhibits an alteration in at least one of the following properties relative to the parent α-amylase:
i) improved pH stability at a pH from 8 to 10.5; and/or
ii) improved Ca^{2+} stability at pH 8 to 10.5, and/or
iii) increased specific activity at temperatures from 10 to 60°C, preferably 20-50°C, especially 30-40°C. Further, details will be described below.

The invention further relates to DNA constructs encoding variants of the invention; to methods for preparing variants of the invention; and to the use of variants of the invention, alone or in combination with other enzymes, in various industrial products or processes, e.g., in detergents or for starch liquefaction.

In a final aspect the invention relates to a method of providing α-amylases with altered pH optimum, and/or altered temperature optimum, and/or improved stability.

Nomenclature

In the present description and claims, the conventional one-

letter and three-letter codes for amino acid residues are used.

For ease of reference, α-amylase variants of the invention are described by use of the following nomenclature:

Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, for instance the substitution of alanine for asparagine in position 30 is shown as:

Ala30Asn or A30N

a deletion of alanine in the same position is shown as:

Ala30* or A30*

and insertion of an additional amino acid residue, such as lysine, is shown as:

Ala30AlaLys or A30AK

A deletion of a consecutive stretch of amino acid residues, such as amino acid residues 30-33, is indicated as (30-33)* or Δ(A30-N33).

Where a specific α-amylase contains a "deletion" in comparison with other α-amylases and an insertion is made in such a position this is indicated as:

*36Asp or *36D

for insertion of an aspartic acid in position 36

Multiple mutations are separated by plus signs, i.e.:

Ala30Asp + Glu34Ser or A30N+E34S

representing mutations in positions 30 and 34 substituting alanine and glutamic acid for asparagine and serine, respectively.

When one or more alternative amino acid residues may be inserted in a given position it is indicated as

A30N,E or

A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine in position 30 is mentioned, but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid, i.e., any one of:

R,N,D,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an alignment of the amino acid sequences of six parent Termamyl-like α-amylases. The numbers on the extreme left designate the respective amino acid sequences as follows:

1: SEQ ID NO: 2
2: Kaoamyl
3: SEQ ID NO: 1
4: SEQ ID NO: 5
5: SEQ ID NO: 4
6: SEQ ID NO: 3.

Figure 2 shows the temperature activity profile of SP722 (SEQ ID NO: 2) (at pH 9) and *B. licheniformis* α-amylase (SEQ ID NO: 4) (at pH 7.3).

Figure 3 shows the temperature profile for SP690 (SEQ ID NO: 1), SP722 (SEQ ID NO: 2), *B. licheniformis* α-amylase (SEQ ID NO: 4) at pH 10.

Figure 4 is an alignment of the amino acid sequences of five α-amylases. The numbers on the extreme left designate the respective amino acid sequences as follows:

1: amyp_mouse
2: amyp_rat
3: amyp_pig porcine pancreatic alpha-amylase (PPA)
4: amyp_human
5: amy_altha *A. haloplanctis* alpha-amylase (AHA)

DETAILED DISCLOSURE OF THE INVENTION

The Termamyl-like α-amylase

It is well known that a number of α-amylases produced by *Bacillus* spp. are highly homologous on the amino acid level. For instance, the *B. licheniformis* α-amylase comprising the amino acid sequence shown in SEQ ID NO:. 4 (commercially available as Termamyl™) has been found to be about 89% homologous with the *B. amyloliquefaciens* α-amylase comprising the amino acid sequence shown in SEQ ID NO: 5 and about 79% homologous with the *B.*

stearothermophilus α-amylase comprising the amino acid sequence shown in SEQ ID NO: 3. Further homologous α-amylases include an α-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO 95/26397, and the α-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31, (see SEQ ID NO: 6).

Still further homologous α-amylases include the α-amylase produced by the *B. licheniformis* strain described in EP 0252666 (ATCC 27811), and the α-amylases identified in WO 91/00353 and WO 94/18314. Other commercial Termamyl-like *B. licheniformis* α-amylases are comprised in the products Optitherm™ and Takatherm™ (available from Solvay), Maxamyl™ (available from Gist-brocades/Genencor), Spezym AA™ and Spezyme Delta AA™ (available from Genencor), and Keistase™ (available from Daiwa).

Because of the substantial homology found between these α-amylases, they are considered to belong to the same class of α-amylases, namely the class of "Termamyl-like α-amylases".

Accordingly, in the present context, the term "Termamyl-like α-amylase" is intended to indicate an α-amylase which, at the amino acid level, exhibits a substantial homology to Termamyl™, i.e., the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID NO:4 herein. In other words, all the following α-amylases which has the amino acid sequences shown in SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7 or 8 herein, or the amino acid sequence shown in SEQ ID NO: 1 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 7 herein) or in SEQ ID NO: 2 of WO 95/26397 (the same as the amino acid sequence shown as SEQ ID NO: 8 herein) or in Tsukamoto et al., 1988, (which amino acid sequence is shown in SEQ ID NO: 6 herein) are considered to be "Termamyl-like α-amylase". Other Termamyl-like α-amylases are α-amylases i) which displays at least 60%, such as at least 70%, e.g., at least 75%, or at least 80%, e.g., at least 85%, at least 90% or at least 95% homology with at least

one of said amino acid sequences shown in SEQ ID NOS: 1-8 and/or ii) displays immunological cross-reactivity with an antibody raised against at least one of said α-amylases, and/or iii) is encoded by a DNA sequence which hybridizes to the DNA sequences encoding the above-specified α-amylases which are apparent from SEQ ID NOS: 9, 10, 11, or 12 of the present application (which encoding sequences encode the amino acid sequences shown in SEQ ID NOS: 1, 2, 3, 4 and 5 herein, respectively), from SEQ ID NO: 4 of WO 95/26397 (which DNA sequence, together with the stop codon TAA, is shown in SEQ ID NO: 13 herein and encodes the amino acid sequence shown in SEQ ID NO: 8 herein) and from SEQ ID NO: 5 of WO 95/26397 (shown in SEQ ID NO: 14 herein), respectively.

In connection with property i), the "homology" may be determined by use of any conventional algorithm, preferably by use of the GAP progamme from the GCG package version 7.3 (June 1993) using default values for GAP penalties, which is a GAP creation penalty of 3.0 and GAP extension penalty of 0.1, (Genetic Computer Group (1991) Programme Manual for the GCG Package, version 7, 575 Science Drive, Madison, Wisconsin, USA 53711).

A structural alignment between Termamyl (SEQ ID NO: 4) and a Termamyl-like α-amylase may be used to identify equivalent/corresponding positions in other Termamyl-like α-amylases. One method of obtaining said structural alignment is to use the Pile Up programme from the GCG package using default values of gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 0.1. Other structural alignment methods include the hydrophobic cluster analysis (Gaboriaud et al., (1987), FEBS LETTERS 224, pp. 149-155) and reverse threading (Huber, T ; Torda, AE, PROTEIN SCIENCE Vol. 7, No. 1 pp. 142-149 (1998).

Property ii) of the α-amylase, i.e., the immunological cross reactivity, may be assayed using an antibody raised against, or reactive with, at least one epitope of the relevant Termamyl-like α-amylase. The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in

the art, e.g., as described by Hudson et al., Practical Immunology, Third edition (1989), Blackwell Scientific Publications. The immunological cross-reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay, e.g., as described by Hudson et al., 1989. In this respect, immunological cross-reactivity between the α-amylases having the amino acid sequences SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, or 8, respectively, has been found.

The oligonucleotide probe used in the characterisation of the Termamyl-like α-amylase in accordance with property iii) above may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the α-amylase in question.

Suitable conditions for testing hybridisation involve presoaking in 5xSSC and prehybridizing for 1 hour at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50mg of denatured sonicated calf thymus DNA, followed by hybridisation in the same solution supplemented with 100mM ATP for 18 hours at ~40°C, followed by three times washing of the filter in 2xSSC, 0.2% SDS at 40°C for 30 minutes (low stringency), preferred at 50°C (medium stringency), more preferably at 65°C (high stringency), even more preferably at ~75°C (very high stringency). More details about the hybridisation method can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989.

In the present context, "derived from" is intended not only to indicate an α-amylase produced or producible by a strain of the organism in question, but also an α-amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended to indicate an α-amylase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the α-amylase in question. The term is also intended to indicate that the parent α-amylase may be a variant of a naturally occurring α-amylase, i.e. a variant which is the result of a modification (insertion, substitution,

deletion) of one or more amino acid residues of the naturally occurring α-amylase.

Parent hybrid α-amylases

The parent α-amylase (i.e., backbone α-amylase) may be a hybrid α-amylase, i.e., an α-amylase which comprises a combination of partial amino acid sequences derived from at least two α-amylases.

The parent hybrid α-amylase may be one which on the basis of amino acid homology and/or immunological cross-reactivity and/or DNA hybridization (as defined above) can be determined to belong to the Termamyl-like α-amylase family. In this case, the hybrid α-amylase is typically composed of at least one part of a Termamyl-like α-amylase and part(s) of one or more other α-amylases selected from Termamyl-like α-amylases or non-Termamyl-like α-amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid α-amylase may comprise a combination of partial amino acid sequences deriving from at least two Termamyl-like α-amylases, or from at least one Termamyl-like and at least one non-Termamyl-like bacterial α-amylase, or from at least one Termamyl-like and at least one fungal α-amylase. The Termamyl-like α-amylase from which a partial amino acid sequence derives may, *e.g.*, be any of those specific Termamyl-like α-amylase referred to herein.

For instance, the parent α-amylase may comprise a C-terminal part of an α-amylase derived from a strain of *B. licheniformis*, and a N-terminal part of an α-amylase derived from a strain of *B. amyloliquefaciens* or from a strain of *B. stearothermophilus*. For instance, the parent α-amylase may comprise at least 430 amino acid residues of the C-terminal part of the *B. licheniformis* α-amylase, and may, *e.g.*, comprise a) an amino acid segment corresponding to the 37 N-terminal amino acid

residues of the *B. amyloliquefaciens* α-amylase having the amino acid sequence shown in SEQ ID NO: 5 and an amino acid segment corresponding to the 445 C-terminal amino acid residues of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID NO: 4, or

a hybrid Termamyl-like α-amylase being identical to the Termamyl sequence, i.e., the *Bacillus licheniformis* α-amylase shown in SEQ ID NO: 4, except that the N-terminal 35 amino acid residues (of the mature protein) has been replaced by the N-terminal 33 residues of BAN (mature protein), i.e., the *Bacillus amyloliquefaciens* α-amylase shown in SEQ ID NO: 5; or b) an amino acid segment corresponding to the 68 N-terminal amino acid residues of the *B. stearothermophilus* α-amylase having the amino acid sequence shown in SEQ ID NO: 3 and an amino acid segment corresponding to the 415 C-terminal amino acid residues of the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID NO: 4.

Another suitable parent hybrid α-amylase is the one previously described in WO 96/23874 (from Novo Nordisk) constituting the N-terminus of BAN, *Bacillus amyloliquefaciens* α-amylase (amino acids 1-300 of the mature protein) and the C-terminus from Termamyl (amino acids 301-483 of the mature protein). Increased activity was achieved by substituting one or more of the following positions of the above hybrid α-amylase (BAN:1-300/Termamyl:301-483): Q360, F290, and N102. Particularly interesting substitutions are one or more of the following substitutions: Q360E,D; F290A,C,D,E,G,H,I,K,L,M,N,P,Q,R,S,T; N102D,E;

The corresponding positions in the SP722 α-amylase shown in SEQ ID NO: 2 are one or more of: S365, Y295, N106. Corresponding substitutions of particular interest in said α-amylase shown in SEQ ID NO: 2 are one or more of: S365D,E; Y295 A,C,D,E,G,H,I,K,L,M,N,P,Q,R,S,T; and N106D,E.

The corresponding positions in the SP690 α-amylase shown in SEQ ID NO: 1 are one or more of: S365, Y295, N106. The

corresponding substitutions of particular interest are one or more of: S365D,E; Y295 A,C,D,E,G,H,I,K,L,M,N,P,Q,R,S,T; N106D,E.

The above mentioned non-Termamyl-like α-amylase may, e.g., be a fungal α-amylase, a mammalian or a plant α-amylase or a bacterial α-amylase (different from a Termamyl-like α-amylase). Specific examples of such α-amylases include the *Aspergillus oryzae* TAKA α-amylase, the *A. niger* acid α-amylase, the *Bacillus subtilis* α-amylase, the porcine pancreatic α-amylase and a barley α-amylase. All of these α-amylases have elucidated structures which are markedly different from the structure of a typical Termamyl-like α-amylase as referred to herein.

The fungal α-amylases mentioned above, i.e., derived from *A. niger* and *A. oryzae*, are highly homologous on the amino acid level and generally considered to belong to the same family of α-amylases. The fungal α-amylase derived from *Aspergillus oryzae* is commercially available under the tradename Fungamyl™.

Furthermore, when a particular variant of a Termamyl-like α-amylase (variant of the invention) is referred to - in a conventional manner - by reference to modification (e.g., deletion or substitution) of specific amino acid residues in the amino acid sequence of a specific Termamyl-like α-amylase, it is to be understood that variants of another Termamyl-like α-amylase modified in the equivalent position(s) (as determined from the best possible amino acid sequence alignment between the respective amino acid sequences) are encompassed thereby.

In a preferred embodiment of the invention the α-amylase backbone is derived from *B. licheniformis* (as the parent Termamyl-like α-amylase), e.g., one of those referred to above, such as the *B. licheniformis* α-amylase having the amino acid sequence shown in SEQ ID NO: 4.

Altered properties of variants of the invention

The following discusses the relationship between mutations which are present in variants of the invention, and desirable

alterations in properties (relative to those a parent Termamyl-like α-amylase) which may result therefrom.

Improved stability at pH 8-10.5

In the context of the present invention, mutations (including amino acid substitutions) of importance with respect to achieving improved stability at high pH (*i.e.*, pH 8-10.5) include mutations corresponding to mutations in one or more of the following positions in SP722 α-amylase (having the amino acid sequence shown in SEQ ID NO: 2): T141, K142, F143, D144, F145, P146, G147, R148, G149, R181, A186, S193, N195, K269, N270, K311, K458, P459, T461.

The variant of the invention have one or more of the following substitutions (using the SEQ ID NO: 2 numbering):

T141A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
K142A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
F143A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
D144A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F145A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
P146A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G147A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
R148A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G149A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
K181A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
A186D,R,N,C,E,Q,G,H,I,L,P,K,M,F,S,T,W,Y,V;
S193A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K269A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N270A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K311A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
K458A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
T461A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V.

Preferred high pH stability variants include one or more of the following substitutions in the SP722 α-amylase (having the amino acid sequence shown in SEQ ID NO: 2):
K142R, R181S, A186T, S193P, N195F, K269R, N270Y, K311R, K458R, P459T and T461P.

In specific embodiments the *Bacillus* strain NCIB 12512 α-amylase having the sequence shown in SEQ ID NO: 1, or the *B. stearothermophilus* α-amylase having the sequence shown in SEQ ID NO: 3, or the *B. licheniformis* α-amylase having the sequence shown in SEQ ID NO: 4, or the *B. amyloliquefaciens* α-amylase having the sequence shown in SEQ ID NO: 5 is used as the backbone, i.e., parent Termamyl-like α-amylase, for these mutations.

As can been seen from the alignment in Figure 1 the *B. stearothermophilus* α-amylase already has a Tyrosine at position corresponding to N270 in SP722. Further, the *Bacillus* strain NCIB 12512 α-amylase, the *B. stearothermophilus* α-amylase, the *B. licheniformis* α-amylase and the *B. amyloliquefaciens* α-amylase already have Arginine at position corresponding to K458 in SP722. Furthermore, the *B. licheniformis* α-amylase already has a Proline at position corresponding to T461 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

α-amylase variants with improved stability at high pH can be constructed by making substitutions in the regions found using the molecular dynamics simulation mentioned in Example 2. The simulation depicts the region(s) that has a higher flexibility or mobility at high pH (i.e., pH 8-10.5) when compared to medium pH.

By using the structure of any bacterial alpha-amylase with homology (as defined below) to the Termamyl-like α-amylase (BA2), of which the 3D structure is disclosed in Appendix 1 of WO 96/23874 (from Novo Nordisk), it is possible to modelbuild the structure of such alpha-amylase and to subject it to molecular dynamics simulations. The homology of said bacterial α-amylase may be at least 60%, preferably be more than 70%, more preferably more than 80%, most preferably more than 90% homologous to the above mentioned Termamyl-like α-amylase (BA2), measured using the UWGCG GAP program from the GCG package version 7.3 (June 1993) using default values for GAP penalties

[Genetic Computer Group (1991) Programme Manual for the GCG Package, version 7, 575 Science Drive, Madison, Wisconsin, USA 53711]. Substitution of the unfavorable residue for another would be applicable.

Improved Ca^{2+} stability at pH 8-10.5

Improved Ca^{2+} stability means the stability of the enzyme under Ca^{2+} depletion has been improved. In the context of the present invention, mutations (including amino acid substitutions) of importance with respect to achieving improved Ca^{2+} stability at high pH include mutation or deletion in one or more positions corresponding to the following positions in the SP722 α-amylase having the amino acid sequence shown in SEQ ID NO: 2: R181, G182, D183, G184, K185, A186, W189, N195, N270, E346, K385, K458, P459.

A variant of the invention have one or more of the following substitutions or deletions:

R181*,A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G182*,A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D183*,A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G184*,A,R,D,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
K185A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
A186D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W189A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
N270A,R,D,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
E346A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K385A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
K458A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,R,D,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V.

Preferred are variants having one or more of the following substitutions or deletions:

R181Q,N; G182T,S,N; D183*; G184*;
K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V; A186T,S,N,I,V;
W189T,S,N,Q; N195F, N270R,D; E346Q; K385R; K458R; P459T.

In specific embodiments the *Bacillus* strain NCIB 12512 α-amylase having the sequence shown in SEQ ID NO: 1, or the *B. amyloliquefaciens* α-amylase having the sequence shown in SEQ ID

NO: 5, or the *B. licheniformis* α-amylase having the sequence shown in SEQ ID NO: 4 are used as the backbone for these mutations.

As can been seen from the alignment in Figure 1 the *B. licheniformis* α-amylase does not have the positions corresponding to D183 and G184 in SP722. Therefore for said α-amylases these deletions are not relevant.

In a preferred embodiment the variant is the *Bacillus* strain NCIB 12512 α-amylase with deletions in D183 and G184 and further one of the following substitutions: R181Q,N and/or G182T,S,N and/or D183*; G184* and/or K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V and/or A186T,S,N,I,V and/or W189T,S,N,Q and/or N195F and/or N270R,D and/or E346Q and/or K385R and/or K458R and/or P459T.

Increased specific activity at medium temperature

In a further aspect of the present invention, important mutations with respect to obtaining variants exhibiting increased specific activity at temperatures from 10-60°C, preferably 20-50°C, especially 30-40°C, include mutations corresponding to one or more of the following positions in the SP722 α-amylase having the amino acid sequence shown in SEQ ID NO: 2:

H107, K108, G109, D166, W167, D168, Q169, S170, R171, Q172, F173, Q174, D183, G184, N195, F267, W268, K269, N270, D271, L272, G273, A274, L275, G456, N457, K458, P459, G460, T461, V462, T463.

The variant of the invention have one or more of the following substitutions:

H107A,D,R,N,C,E,Q,G,I,L,K,M,F,P,S,T,W,Y,V;
K108A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G109A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D166A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W167A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
D168A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q169A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;

S170A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
R171A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q172A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F173A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
Q174*,A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D183*,A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
G184*,A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F267A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
W268A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
K269A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N270A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D271A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L272A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
G273A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
A274D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L275A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
G456A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
N457A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K458A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G460A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
T461A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
V462A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
T463A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V.

Preferred variants has one or more of the following substitutions or deletions: Q174*, D183*, G184*, K269S.

In a specific embodiment the *B. licheniformis* α-amylase having the sequence shown in SEQ ID NO: 4 is used as the backbone for these mutations.

General mutations in variants of the invention: increased specific activity at medium temperatures

The particularly interesting amino acid substitution are those that increase the mobility around the active site of the enzyme. This is accomplished by changes that disrupt stabilizing interaction in the vicinity of the active site, *i.e.*, within preferably 10Å or 8Å or 6Å or 4Å from any of the residues

constituting the active site.

Examples are mutations that reduce the size of side chains, such as

Ala to Gly,

Val to Ala or Gly,

Ile or Leu to Val, Ala, or Gly

Thr to Ser

Such mutations are expected to cause increased flexibility in the active site region either by the introduction of cavities or by the structural rearrangements that fill the space left by the mutation.

It may be preferred that a variant of the invention comprises one or more modifications in addition to those outlined above. Thus, it may be advantageous that one or more Proline residues present in the part of the α-amylase variant which is modified is/are replaced with a non-Proline residue which may be any of the possible, naturally occurring non-Proline residues, and which preferably is an Alanine, Glycine, Serine, Threonine, Valine or Leucine.

Analogously, it may be preferred that one or more Cysteine residues present among the amino acid residues with which the parent α-amylase is modified is/are replaced with a non-Cysteine residue such as Serine, Alanine, Threonine, Glycine, Valine or Leucine.

Furthermore, a variant of the invention may - either as the only modification or in combination with any of the above outlined modifications - be modified so that one or more Asp and/or Glu present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 is replaced by an Asn and/or Gln, respectively. Also of interest is the replacement, in the Termamyl-like α-amylase, of one or more of the Lys residues present in an amino acid fragment corresponding to the amino acid fragment 185-209 of SEQ ID NO: 4 by an Arg.

It will be understood that the present invention encompasses variants incorporating two or more of the above outlined modifications.

Furthermore, it may be advantageous to introduce point-mutations in any of the variants described herein.

α-amylase variants having increased mobility around the active site:

The mobility of α-amylase variants of the invention may be increased by replacing one or more amino acid residue at one or more positions close to the substrate site. These positions are (using the SP722 α-amylase (SEQ ID NO: 2) numbering): V56, K108, D168, Q169, Q172, L201, K269, L272, L275, K446, P459.

Therefore, in an aspect the invention relates to variants being mutated in one or more of the above mentioned positions.

Preferred substitutions are one or more of the following:
V56A,G,S,T;
K108A,D,E,Q,G,H,I,L,M,N,S,T,V;
D168A,G,I,V,N,S,T;
Q169A,D,G,H,I,L,M,N,S,T,V;
Q172A,D,G,H,I,L,M,N,S,T,V;
L201A,G,I,V,S,T;
K269A,D,E,Q,G,H,I,L,M,N,S,T,V;
L272A,G,I,V,S,T;
L275A,G,I,V,S,T;
Y295A,D,E,Q,G,H,I,L,M,N,F,S,T,V;
K446A,D,E,Q,G,H,I,L,M,N,S,T,V;
P459A,G,I,L,S,T,V.

In specific embodiments of the invention the *Bacillus* strain NCIB 12512 α-amylase having the sequence shown in SEQ ID NO: 1, or *the B. stearothermophilus* α-amylase having the sequence shown in SEQ ID NO: 3, or the *B. licheniformis* α-amylase having the sequence shown in SEQ ID NO: 4, or the *B. amyloliquefaciens* α-amylase having the sequence shown in SEQ ID NO: 5 are used as the backbone for these mutations.

As can been seen from the alignment in Figure 1 *the B. licheniformis* α-amylase and the *B. amyloliquefaciens* α-amylase have a Glutamine at position corresponding to K269 in SP722. Further, the *B. stearothermophilus* α-amylase has a Serine at position corresponding to K269 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

Furthermore, as can been seen from the alignment in Figure 1

the *B. amyloliquefaciens* α-amylase has an Alanine at position corresponding to L272 in SP722, and the *B. stearothermophilus* α-amylase has a Isoleucine at the position corresponding to L272 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

As can been seen from the alignment in Figure 1, the *Bacillus* strain 12512 α-amylase has a Isoleucine at position corresponding to L275 in SP722. Therefore for said α-amylase this substitution is not relevant.

As can been seen from the alignment in Figure 1 *the B. amyloliquefaciens* α-amylase has a Phenylalanine at position corresponding to Y295 in SP722. Further, *the B. stearothermophilus* α-amylase has an Asparagine at position corresponding to Y295 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

As can been seen from the alignment in Figure 1 *the B. licheniformis* α-amylase and the *B. amyloliquefaciens* α-amylase have a Asparagine at position corresponding to K446 in SP722. Further, the *B. stearothermophilus* α-amylase has a Histidine at position corresponding to K446 in SP722. Therefore, for said a-amylases these substitutions are not relevant.

As can been seen from the alignment in Figure 1 the *B. licheniformis* α-amylase, the *B. amyloliquefaciens* α-amylase and the *B. stearothermophilus* α-amylase have a Serine at position corresponding to P459 in SP722. Further, the *Bacillus* strain 12512 α-amylase has a Threonine at position corresponding to P459 in SP722. Therefore, for said α-amylases these substitutions are not relevant.

Stabilization of enzymes having high activity at medium temperatures

In a further embodiment the invention relates to improving the stability of low temperature α-amylases (e.g, *Alteromonas haloplanctis* (Feller et al., (1994), Eur. J. Biochem 222:441-447), and medium temperature α-amylases (e.g., SP722 and SP690) possessing medium temperature activity, i.e., commonly known as psychrophilic enzymes and mesophilic enzymes. The stability can

for this particular enzyme class be understood either as thermostability or the stability at Calcium depletion conditions.

Typically, enzymes displaying the high activity at medium temperatures also display severe problems under conditions that stress the enzyme, such as temperature or Calcium depletion.

Consequently, the objective is to provide enzymes that at the same time display the desired high activity at medium temperatures without loosing their activity under slightly stressed conditions.

The activity of the stabilized variant measured at medium temperatures should preferably be between 100% or more and 50%, and more preferably between 100% or more and 70%, and most preferably between 100% or more and 85% of the original activity at that specific temperature before stabilization of the enzyme and the resulting enzyme should withstand longer incubation at stressed condition than the wild type enzyme.

Contemplated enzymes include α-amylases of, *e.g.*, bacterial or fungal origin.

An example of such a low temerature α-amylase is the one isolated from *Alteromonas haloplanctis* (Feller et al., (1994), Eur. J. Biochem 222:441-447). The crystal structure of this alpha-amylase has been solved (Aghajari et al., (1998), Protein Science 7:564-572).

The *A. haloplanctis* alpha-amylase (5 in alignment shown in Fig. 4) has a homology of approximately 66% to porcine pancreatic alpha-amylase (PPA) (3 in the alignment shown in Fig. 4). The PPA 3D structure is known, and can be obtained from Brookhaven database under the name 1OSE or 1DHK. Based on the homology to other more stable alpha amylases, stabilization of "the low temperature highly active enzyme" from *Alteromonas haloplanctis* alpha-amylase, can be obtained and at the same time retaining the desired high activity at medium temperatures.

Figure 4 shown a multiple sequence alignments of five α-amylases, including the AHA and the PPA α-amylase. Specific mutations giving increased stability in *Alteromonas haloplantis* alpha-amylase:

T66P, Q69P, R155P, Q177R, A205P, A232P, L243R, V295P, S315R.

Methods for preparing α-amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of α-amylase-encoding DNA sequences, methods for generating mutations at specific sites within the α-amylase-encoding sequence will be discussed.

Cloning a DNA sequence encoding an α-amylase

The DNA sequence encoding a parent α-amylase may be isolated from any cell or microorganism producing the α-amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α-amylase to be studied. Then, if the amino acid sequence of the α-amylase is known, homologous, labeled oligonucleotide probes may be synthesized and used to identify α-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labeled oligonucleotide probe containing sequences homologous to a known α-amylase gene could be used as a probe to identify α-amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying α-amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for α-amylase, thereby allowing clones expressing the α-amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g., the phosphoroamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoroamidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Expression of α-amylase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an α-amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α-amylase variant of the invention,

sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an α-amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g., when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* α-amylases mentioned herein comprise a preregion permitting secretion of the expressed protease into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention encoding an α-amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an α-amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g., by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are Gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus*

thuringiensis, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g., *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing an α-amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the α-amylase variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The α-amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Industrial Applications

The α-amylase variants of this invention possesses valuable properties allowing for a variety of industrial applications. In particular, enzyme variants of the invention are applicable as a component in washing, dishwashing and hard-surface cleaning

detergent compositions.

Numerous variants are particularly useful in the production of sweeteners and ethanol from starch, and/or for textile desizing. Conditions for conventional starch- conversion processes, including starch liquefaction and/or saccharification processes, are described in, *e.g.*, US 3,912,590 and in EP patent publications Nos. 252,730 and 63,909.

Detergent compositions

As mentioned above, variants of the invention may suitably be incorporated in detergent compositions. Reference is made, for example, to WO 96/23874 and WO 97/07202 for further details concerning relevant ingredients of detergent compositions (such as laundry or dishwashing detergents), appropriate methods of formulating the variants in such detergent compositions, and for examples of relevant types of detergent compositions.

Detergent compositions comprising a variant of the invention may additionally comprise one or more other enzymes, such as a lipase, cutinase, protease, cellulase, peroxidase or laccase, and/or another α-amylase.

α-amylase variants of the invention may be incorporated in detergents at conventionally employed concentrations. It is at present contemplated that a variant of the invention may be incorporated in an amount corresponding to 0.00001-1 mg (calculated as pure, active enzyme protein) of α-amylase per liter of wash/dishwash liquor using conventional dosing levels of detergent.

The invention also relates to a method of providing α-amylases with 1) altered pH optimum, and/or 2) altered temperature optimum, and/or 3) improved stability, comprising the following steps:

i) identifying (a) target position(s) and/or region(s) for mutation of the α-amylase by comparing the molecular dynamics of two or more α-amylase 3D structures having substantially different pH, temperature and/or stability profiles,

ii) substituting, adding and/or deleting one or more amino acids in the identified position(s) and/or region(s).

In embodiment of the invention a medium temperature α-amylase is compared with a high temperature α-amylase. In another embodiment a low temperature α-amylase is compared with either a medium or a high temperature α-amylase.

The α-amylases compared should preferably be at least 70%, preferably 80%, up to 90%, such as up to 95%, especially 95% homologous with each other.

The α-amylases compared may be Termamyl-like α-amylases as defined above. In specific embodiment the α-amylases compared are the α-amylases shown in SEQ ID NO: 1 to SEQ ID NO: 8.

In another embodiment the stability profile of the α-amylases in question compared are the Ca^{2+} dependency profile.

MATERIALS AND METHODS

Enzymes:

SP722: (SEQ ID NO: 2, available from Novo Nordisk)
Termamyl™ (SEQ ID NO: 4, available from Novo Nordisk)
SP690: (SEQ ID NO: 1, available from Novo Nordisk)

Bacillus subtilis SHA273: see WO 95/10603

Plasmids

pJE1 contains the gene encoding a variant of SP722 α-amylase (SEQ ID NO: 2): viz. deletion of 6 nucleotides corresponding to amino acids D183-G184 in the mature protein. Transcription of the JE1 gene is directed from the *amyL* promoter. The plasmid further more contains the origin of replication and *cat*-gene conferring resistance towards kanamycin obtained from plasmid pUB110 (Gryczan, TJ et al. (1978), J. Bact. 134:318-329).

Methods:

Construction of library vector pDorK101

The *E. coli/Bacillus* shuttle vector pDorK101 (described below) can be used to introduce mutations without expression of α-amylase in *E. coli* and then be modified in such way that the α-amylase is active in Bacillus. The vector was constructed as

follows: The JE1 encoding gene (SP722 with the deletion of D183-G184) was inactivated in pJE1 by gene interruption in the PstI site in the 5´coding region of the SEQ ID NO: 2: SP722 by a 1.2 kb fragment containing an *E. coli* origin of replication. This fragment was PCR amplified from the pUC19 (GenBank Accession #:X02514) using the forward primer: 5´-gacctgcagtcaggcaacta-3´ and the reverse primer: 5´-tagagtcgacctgcaggcat-3´. The PCR amplicon and the pJE1 vector were digested with PstI at 37°C for 2 hours. The pJE1 vector fragment and the PCR fragment were ligated at room temperature. for 1 hour and transformed in *E. coli* by electrotransformation. The resulting vector is designated pDorK101.

Filter screening assays

The assay can be used to screening of Termamyl-like α-amylase variants having an improved stability at high pH compared to the parent enzyme and Termamyl-like α-amylase variants having an improved stability at high pH and medium temperatures compared to the parent enzyme depending of the screening temperature setting

High pH filter assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with 10 µg/ml kanamycin at 37°C for at least 21 hours. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with glycin-NaOH buffer, pH 8.6-10.6 and incubated at room temperature(can be altered from 10°-60°C) for 15 min. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on plates containing 1% agarose,

0.2% starch in glycin-NaOH buffer, pH 8.6-10.6. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours. at room temperature. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

Low calcium filter assay

The *Bacillus* library are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany) - and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with a relevant antibiotic, *e.g.*, kanamycin or chloramphenicol, at 37°C for at least 21 hours. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with carbonate/bicarbonate buffer pH 8.5-10 and with different EDTA concentrations (0.001 mM - 100 mM). The filters are incubated at room temperature for 1 hour. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on plates containing 1% agarose, 0.2% starch in carbonate/bicarbonate buffer pH 8.5-10. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours. at room temperature. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

Method for obtaining the regions of interest:

There are three known 3D structures of bacterial α-

amylases. Two of *B. licheniformis* α-amylase, Brookhaven database 1BPL (Machius et al. (1995), J. Mol. Biol. 246, p. 545-559) and 1VJS (Song et al. (1996), Enzymes for Carbohydrate 163 Engineering (Prog. Biotechnol. V 12). These two structures are lacking an important piece of the structure from the so-called B-domain, in the area around the two Calcium ions and one Sodium ion binding sites. We have therefore used a 3D structure of an α-amylase BA2 (WO 96/23874 which are a hybrid between BAN™ (SEQ ID NO. 5) and *B. licheniformis* α-amylase (SEQ ID NO. 4). On basis of the structure a model of *B. licheniformis* alpha amylase and the SP722 α-amylase has been build.

Fermentation and purification of α-amylase variants

Fermentation and purification may be performed by methods well known in the art.

Stability determination

All stability trials are made using the same set up. The method are:

The enzyme is incubated under the relevant conditions (1-4). Samples are taken at various time points, e.g., after 0, 5, 10, 15 and 30 minutes and diluted 25 times (same dilution for all taken samples) in assay buffer (0.1M 50mM Britton buffer pH 7.3) and the activity is measured using the Phadebas assay (Pharmacia) under standard conditions pH 7.3, 37°C.

The activity measured before incubation (0 minutes) is used as reference (100%). The decline in percent is calculated as a function of the incubation time. The table shows the residual activity after, e.g., 30 minutes of incubation.

Specific activity determination

The specific activity is determined using the Phadebas assay (Pharmacia) as activity/mg enzyme. The manufactures instructions are followed (see also below under "Assay for α-amylase activity).

Assays for α-Amylase Activity

1. Phadebas assay

α-amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-colored starch polymer which has been mixed with bovine serum albumin and a buffer substance and tabletted.

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM $CaCl_2$, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The α-amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this α-amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolyzed by the α-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the α-amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given α-amylase will hydrolyze a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure α-amylase protein) of the α-amylase in question under the given set of conditions.

2. Alternative method

α-amylase activity is determined by a method employing the PNP-G7 substrate. PNP-G7 which is a abbreviation for p-nitrophenyl-α,D-maltoheptaoside is a blocked oligosaccharide which can be cleaved by an endo-amylase. Following the cleavage,

the α-Glucosidase included in the kit digest the substrate to liberate a free PNP molecule which has a yellow colour and thus can be measured by visible spectophometry at λ=405nm. (400-420 nm.). Kits containing PNP-G7 substrate and α-Glucosidase is manufactured by Boehringer-Mannheim (cat.No. 1054635).

To prepare the substrate one bottle of substrate (BM 1442309) is added to 5 ml buffer (BM1442309). To prepare the α-Glucosidase one bottle of α-Glucosidase (BM 1462309) is added to 45 ml buffer (BM1442309). The working solution is made by mixing 5 ml α-Glucosidase solution with 0.5 ml substrate.

The assay is performed by transforming 20µl enzyme solution to a 96 well microtitre plate and incubating at 25°C. 200 µl working solution, 25°C is added. The solution is mixed and pre-incubated 1 minute and absorption is measured every 15 sec. over 3 minutes at OD 405 nm.

The slope of the time dependent absorption-curve is directly proportional to the specific activity (activity per mg enzyme) of the α-amylase in question under the given set of conditions.

General method for random mutagenesis by use of the DOPE program

The random mutagenesis may be carried out by the following steps:

1. Select regions of interest for modification in the parent enzyme
2. Decide on mutation sites and non-mutated sites in the selected region
3. Decide on which kind of mutations should be carried out, e.g. with respect to the desired stability and/or performance of the variant to be constructed
4. Select structurally reasonable mutations.
5. Adjust the residues selected by step 3 with regard to step 4.
6. Analyze by use of a suitable dope algorithm the nucleotide distribution.
7. If necessary, adjust the wanted residues to genetic code realism (e.g., taking into account constraints resulting from

the genetic code (e.g. in order to avoid introduction of stop codons))(the skilled person will be aware that some codon combinations cannot be used in practice and will need to be adapted)

8. Make primers
9. Perform random mutagenesis by use of the primers
10. Select resulting α-amylase variants by screening for the desired improved properties.

Suitable dope algorithms for use in step 6 are well known in the art. One algorithm is described by Tomandl, D. et al., Journal of Computer-Aided Molecular Design, 11 (1997), pp. 29-38). Another algorithm, DOPE, is described in the following:

The dope program

The "DOPE" program is a computer algorithm useful to optimize the nucleotide composition of a codon triplet in such a way that it encodes an amino acid distribution which resembles most the wanted amino acid distribution. In order to assess which of the possible distributions is the most similar to the wanted amino acid distribution, a scoring function is needed. In the "Dope" program the following function was found to be suited:

$$s \equiv \prod_{i=1}^{N} \left(\frac{x_i^{y_i}}{y_i^{y_i}} \frac{(1-x_i)^{1-y_i}}{(1-y_i)^{1-y_i}} \right)^{w_i},$$

where the x_i's are the obtained amounts of amino acids and groups of amino acids as calculated by the program, y_i's are the wanted amounts of amino acids and groups of amino acids as defined by the user of the program (e.g. specify which of the 20 amino acids or stop codons are wanted to be introduced, e.g. with a certain percentage (e.g. 90% Ala, 3% Ile, 7% Val), and w_i's are assigned weight factors as defined by the user of the program (e.g., depending on the importance of having a specific amino acid residue inserted into the position in question). N is 21 plus the number of amino acid groups as defined by the

user of the program. For purposes of this function 0^0 is defined as be

A Monte-Carlo algorithm (one example being the one described by Valleau, J.P. & Whittington, S.G. (1977) A guide to Mont Carlo for statistical mechanics: 1 Highways. In "Stastistical Mechanics, Part A" Equlibrium Techniqeues ed. B.J. Berne, New York: Plenum) is used for finding the maximum value of this function. In each iteration the following steps are performed:

1. A new random nucleotide composition is chosen for each base, where the absolute difference between the current and the new composition is smaller than or equal to *d* for each of the four nucleotides G,A,T,C in all three positions of the codon (see below for definition of *d*).
2. The scores of the new composition and the current composition are compared by the use of the function s as described above. If the new score is higher or equal to the score of the current composition, the new composition is kept and the current composition is changed to the new one. If the new score is smaller, the probability of keeping the new composition is $\exp(1000(new\_score - current\_score))$.

A cycle normally consists of 1000 iterations as described above in which *d* is decreasing linearly from 1 to 0. One hundred or more cycles are performed in an optimization process. The nucleotide composition resulting in the highest score is finally presented.

EXAMPLES

EXAMPLE 1

Example on Homology building of Termamyl™

The overall homology of the *B. licheniformis* α-amylase (in the following referred to as Termamyl™) to other Termamyl-like α-amylases is high and the percent similarity is extremely high. The similarity calculated for Termamyl™ to BSG (the *B. stearothermophilus* α-amylase having SEQ ID NO: 3), and BAN (the *B. amyloliquefaciens* α-amylase having SEQ ID NO: 5) using the

University of Wisconsin Genetics Computer Group's program GCG gave 89% and 78%, respectively. TERM has a deletion of 2 residues between residue G180 and K181 compared to BAN™ and BSG. BSG has a deletion of 3 residues between G371 and I372 in comparison with BAN™ and Termamyl™. Further BSG has a C-terminal extension of more than 20 residues compared to BAN™ and Termamyl™. BAN™ has 2 residues less and Termamyl has one residue less in the N-terminal compared to BSG.

The structure of the *B. licheniformis* (Termamyl™) and of the *B. amyloliquefaciens* α-amylase (BAN™), respectively, was model built on the structure disclosed in Appendix 1 of WO 96/23974. The structure of other Termamyl-like α-amylases (*e.g.* those disclosed herein) may be built analogously.

In comparison with the α-amylase used for elucidating the present structure, Termamyl™ differs in that it lacks two residues around 178-182. In order to compensate for this in the model structure, the HOMOLOGY program from BIOSYM was used to substitute the residues in equivalent positions in the structure (not only structurally conserved regions) except for the deletion point. A peptide bond was established between G179(G177) and K180(K180) in Termamyl™(BAN™). The close structural relationship between the solved structure and the model structure (and thus the validity of the latter) is indicated by the presence of only very few atoms found to be too close together in the model.

To this very rough structure of Termamyl™ was then added all waters (605) and ions (4 Calcium and 1 Sodium) from the solved structure (See Appendix 1 of WO 96/23874) at the same coordinates as for said solved structure using the INSIGHT program. This could be done with only few overlaps - in other words with a very nice fit. This model structure were then minimized using 200 steps of Steepest descent and 600 steps of Conjugated gradient (see Brooks et al 1983, J. Computational Chemistry 4, p.187-217). The minimized structure was then subjected to molecular dynamics, 5ps heating followed by up to 200ps equilibration but more than 35ps. The dynamics as run with the Verlet algorithm and the equilibration temperature 300K were kept using the Behrendsen coupling to a water bath (Berendsen

et. al., 1984, J. Chemical Physics 81, p. 3684-3690). Rotations and translations were removed every pico second.

EXAMPLE 2

Method of extracting important regions for identifying α-amylase variants with improved pH stability and altered temperature activity

The X-ray structure and/or the model build structure of the enzyme of interest, here SP722 and Termamyl™, are subjected to molecular dynamics simulations. The molecular dynamics simulation are made using the CHARMM (from Molecular simulations (MSI)) program or other suited program like, e.g., DISCOVER (from MSI). The molecular dynamic analysis is made in vacuum, or more preferred including crystal waters, or with the enzyme embedded in water, *e.g.*, a water sphere or a water box. The simulation are run for 300 pico seconds (ps) or more, *e.g.*, 300-1200 ps. The isotropic fluctuations are extracted for the CA carbons of the structures and compared between the structures. Where the sequence has deletions and/or insertions the isotropic fluctuations from the other structure are inserted thus giving 0 as difference in isotropic fluctuation. For explanation of isotropic fluctuations see the CHARMM manual (obtainable from MSI).

The molecular dynamics simulation can be done using standard charges on the chargeable amino acids. This is Asp and Glu are negatively charged and Lys and Arg are positively charged. This condition resembles the medium pH of approximately 7. To analyze a higher or lower pH, titration of the molecule can be done to obtain the altered pKa's of the standard titrateable residues normally within pH 2-10; Lys, Arg, Asp, Glu, Tyr and His. Also Ser, Thr and Cys are titrateable but are not taking into account here. Here the altered charges due to the pH has been described as both Asp and Glu are negative at high pH, and both Arg and Lys are uncharged. This imitates a pH around 10 to 11 where the titration of Lys and Arg starts, as the normal pKa of these residues are around 9-11.

1. The approach used for extracting important regions for identifying α-amylase variants with high pH stability:

The important regions for constructing variants with improved pH stability are the regions which at the extreme pH display the highest mobility, i.e., regions having the highest isotropic fluctuations.

Such regions are identified by carrying out two molecular dynamics simulations: i) a high pH run at which the basic amino acids, Lys and Arg, are seen as neutral (i.e. not protonated) and the acidic amino acids, Asp and Glu, have the charge (-1) and ii) a neutral pH run with the basic amino acids, Lys and Arg, having the net charge of (+1) and the acidic amino acids having a charge of (-1).

The two run are compared and regions displaying the relatively higher mobility at high pH compared to neutral pH analysis were identified.

Introduction of residues improving general stability, e.g., hydrogen bonding, making the region more rigid (by mutations such as Proline substitutions or replacement of Glycine residues), or improving the charges or their interaction, improves the high pH stability of the enzyme.

2. The approach used for extracting regions for identifying α-amylase variants with increased activity at medium temperatures:

The important regions for constructing variants with increased activity at medium temperature was found as the difference between the isotropic fluctuations in SP722 and Termamyl, i.e., SP722 minus Termamyl CA isotrophic fluctuations, The regions with the highest mobility in the isotrophic fluctuations were selected. These regions and there residues were expected to increase the activity at medium temperatures. The activity of an alpha-amylase is only expressed if the correct mobility of certain residues are present. If the mobility of the residues is too low the activity is decreased or abandoned.

EXAMPLE 3

Construction, by localized random, doped mutagenesis, of Termamyl-like α-amylase variants having an improved Ca2+ stability at medium temperatures compared to the parent enzyme

To improve the stability at low calcium concentration of α-amylases random mutagenesis in pre-selected region was performed.

Region:	Residue:
SAI:	R181-W189

The DOPE software (see Materials and Methods) was used to determine spiked codons for each suggested change in the SA1 region minimizing the amount of stop codons (see table 1). The exact distribution of nucleotides was calculated in the three positions of the codon to give the suggested population of amino acid changes. The doped regions were doped specifically in the indicated positions to have a high chance of getting the desired residues, but still allow other possibilities.

Table 1:
Distribution of amino acid residues for each position
R181: 72% R, 2% N, 7% Q, 4% H, 4%K, 11%S
G182: 73% G, 13% A, 12% S, 2% T
K185: 95% K, 5% R
A186: 50% A, 4% N, 6% D, 1%E, 1% G, 1% K, 5% S, 31% T
W187: 100% W
D188: 100% D
W189: 92% W, 8% S

The resulting doped oligonucleotide strand is shown in table 2 as sense strand: with the wild type nucleotide and amino acid sequences and the distribution of nucleotides for each doped position.

Table 2:

Position	181	182	185	186	187	188	189
Amino acid seq.	Arg	Gly	Lys	Ala	Thr	Asp	Thr
Wt nuc. seq.	cga	ggt	aaa	gct	tgg	gat	tgg

Forward primer (SEQ ID NO: 15):
FSA: 5′-caa aat cgt atc tac aaa ttc 123 456 a7g 8910 tgg gat t11g gaa gta gat tcg gaa aat-3′

Distribution of nucleotides for each doped Position
1: 35% A, 65% C
2: 83% G, 17% A

3: 63% G, 37% T
4: 86% G, 14% A
5: 85% G, 15% C
6: 50% T, 50% C
7: 95% A, 5%G
8: 58% G, 37% A, 5% T
9: 86% C, 13% A, 1% G
10: 83% T, 17% G
11: 92% G, 8% C

Reverse primer (SEQ ID NO: 16):
RSA: 5´-gaa ttt gta gat acg att ttg-3´

Random mutagenesis

The spiked oligonucleotides apparent from Table 2 (which by a common term is designated FSA) and reverse primers RSA for the SA1 region and specific SEQ ID NO: 2: SP722 primers covering the SacII and the DraIII sites are used to generate PCR-library-fragments by the overlap extension method (Horton et al., Gene, 77 (1989), pp. 61-68) with an overlap of 21 base pairs. Plasmid pJE1 is template for the Polymerase Chain Reaction. The PCR fragments are cloned in the *E. coli/Bacillus* shuttle vector pDork101 (see Materials and Methods) enabling mutagenesis in *E. coli* and immediate expression in *Bacillus subtilis* preventing lethal accumulation of amylases in *E. coli*. After establishing the cloned PCR fragments *in E. coli*, a modified pUC19 fragment is digested out of the plasmid and the promoter and the mutated Termamyl gene is physically connected and expression can take place in *Bacillus*.

Screening

The library may be screened in the low calcium filter assays described in the "Material and Methods" section above.

EXAMPLE 4

Construction of variants of amylase SEQ ID NO: 1 (SP690)

The gene encoding the amylase from SEQ ID NO: 1 is located in a plasmid pTVB106 described in WO96/23873. The amylase is expressed from the amyL promoter in this construct in *Bacillus subtilis*.

A variant of the protein is delta(T183-G184) +Y243F+Q391E+K444Q. Construction of this variant is described

in WO96/23873.

Construction of delta(T183-G184) + N195F by the mega-primer method as described by Sarkar and Sommer, (1990), BioTechniques 8: 404-407.

Gene specific primer B1 (SEQ ID NO: 17) and mutagenic primer 101458 (SEQ ID NO: 19) were used to amplify by PCR an approximately 645 bp DNA fragment from a pTVB106-like plasmid (with the delta(T183-G184) mutations in the gene encoding the amylase from SEQ ID NO: 1).

The 645 bp fragment was purified from an agarose gel and used as a mega-primer together with primer Y2 (SEQ ID NO: 18) in a second PCR carried out on the same template.

The resulting approximately 1080 bp fragment was digested with restriction enzymes BstEII and AflIII and the resulting approximately 510 bp DNA fragment was purified and ligated with the pTVB106-like plasmid (with the delta(T183-G184) mutations in the gene encoding the amylase from SEQ ID NO: 1) digested with the same enzymes. Competent *Bacillus subtilis* SHA273 (amylase and protease low) cells were transformed with the ligation and Chlorampenicol resistant transformants and was checked by DNA sequencing to verify the presence of the correct mutations on the plasmid.

primer B1: (SEQ ID NO: 17)

5' CGA TTG CTG ACG CTG TTA TTT GCG 3'

primer Y2: (SEQ ID NO: 18)

5' CTT GTT CCC TTG TCA GAA CCA ATG 3'

primer 101458 (SEQ ID NO: 19):

5' GT CAT AGT TGC CGA AAT CTG TAT CGA CTT C 3'

The construction of variant: delta(T183-G184) + K185R+A186T was carried out in a similar way except that mutagenic primer 101638 was used.

primer 101638: (SEQ ID NO: 20)

5' CC CAG TCC CAC GTA CGT CCC CTG AAT TTA TAT ATT TTG 3'

Variants: delta(T183-G184) +A186T, delta(T183-G184) +A186I, delta(T183-G184) +A186S, delta(T183-G184) +A186N are constructed by a similar method except that pTVB106-like plasmid (carrying variant delta(T183-G184) + K185R+A186T) is used as template and as the vector for the cloning purpose. The

mutagenic oligonucleotide (Oligo 1) is:

5′ CC CAG TCC CAG NTCTTT CCC CTG AAT TTA TAT ATT TTG 3′ (SEQ ID NO: 21)

N represents a mixture of the four bases: A, C, G, and T used in the synthesis of the mutagenicoli-gonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 186 in the mature amylase.

Variant: delta(T183-G184) + K185R+A186T+N195F is constructed as follows:

PCR is carried out with primer x2 (SEQ ID NO: 22) and primer 101458 (SEQ ID NO: 19) on pTVB106-like plasmid (with mutations delta(T183-G184) + K185R+A186T). The resulting DNA fragment is used as a mega-primer together with primer Y2 (SEQ ID NO: 18) in a PCR on pTVB106-like plasmid (with mutations delta(T183-G184) + N195). The product of the second PCR is digested with restriction endonucleases Acc65I and AflIII and cloned into pTVB106 like plasmid (delta(T183-G184)+N195F) digested with the same enzymes.

primer x2: (SEQ ID NO: 22)

5′ GCG TGG ACA AAG TTT GAT TTT CCT G 3′

Variant: delta(T183-G184) + K185R+A186T+N195F+Y243F+Q391E+K444Q is constructed as follows:

PCR is carried out with primer x2 and primer 101458 on pTVB106-like plasmid (with mutations delta(T183-G184) + K185R+A186T). The resulting DNA fragment is used as a mega-primer together with primer Y2 in a PCR on pTVB106 like plasmid (with mutations delta(T183-G184) +Y243F+Q391E+K444Q). The product of the second PCR is digested with restriction endonucleases Acc65I and AflIII and cloned into pTVB106 like plasmid (delta(T183-G184) +Y243F+Q391E+K444Q) digested with the same enzymes.

Example 5

Construction of site-directed α-amylase variants in the parent SP722 α-amylase (SEQ ID NO: 2)

Construction of variants of amylase SEQ ID NO: 2 (SP722) is carried out as described below.

The gene encoding the amylase from SEQ ID NO: 2 is located

in a plasmid pTVB112 described in WO 96/23873. The amylase is expressed from the amyL promoter in this construct *in Bacillus subtilis*.

Construction of delta(D183-G184) + V56I by the mega-primer method as described by Sarkar and Sommer, 1990 (BioTechniques 8: 404-407).

Gene specific primer DA03 and mutagenic primer DA07 are used to amplify by PCR an approximately 820 bp DNA fragment from a pTVB112-like plasmid (with the delta(D183-G184) mutations in the gene encoding the α-amylase shown in SEQ ID NO: 2.

The 820 bp fragment is purified from an agarose gel and used as a mega-primer together with primer DA01 in a second PCR carried out on the same template.

The resulting approximately 920 bp fragment is digested with restriction enzymes NgoM I and Aat II and the resulting approximately 170 bp DNA fragment is purified and ligated with the pTVB112-like plasmid (with the delta(D183-G184) mutations in the gene encoding the amylase shown in SEQ ID NO: 2) digested with the same enzymes. Competent *Bacillus subtilis* SHA273 (amylase and protease low) cells are transformed with the ligation and Chlorampenicol resistant transformants are checked by DNA sequencing to verify the presence of the correct mutations on the plasmid.

primer DA01: (SEQ ID NO: 23)
5′ CCTAATGATGGGAATCACTGG 3′
primer DA03: (SEQ ID NO:24)
5′ GCATTGGATGCTTTTGAACAACCG 3′
primer DA07 (SEQ ID NO:25):
5′ CGCAAAATGATATCGGGTATGGAGCC 3′

Variants: delta(D183-G184) + K108L, delta(D183-G184) + K108Q, delta(D183-G184) + K108E, delta(D183-G184) + K108V, were constructed by the mega-primer method as described by Sarkar and Sommer ,1990 (BioTechniques 8: 404-407):

PCR is carried out with primer DA03 and mutagenesis primer DA20 on pTVB112-like plasmid (with mutations delta(D183-G184)). The resulting DNA fragment is used as a mega-primer together with primer DA01 in a PCR on pTVB112-like plasmid (with

mutations delta(D183-G184)). The approximately 920 bp product of the second PCR is digested with restriction endonucleases Aat II and Mlu I and cloned into pTVB112-like plasmid (delta(D183-G184)) digested with the same enzymes.

primer DA20 (SQ ID NO:26):

5' GTGATGAACCACSWAGGTGGAGCTGATGC 3'

S represents a mixture of the two bases: C and G used in the synthesis of the mutagenic oligonucleotide and W represents a mixture of the two bases: A and T used in the synthesis of the mutagenic oligonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 108 in the mature amylase.

Construction of the variants: delta(D183-G184) + D168A, delta(D183-G184) + D168I, delta(D183-G184) + D168V, delta(D183-G184) + D168T is carried out in a similar way except that mutagenic primer DA14 is used.

primer DA14 (SEQ ID NO:27):

5' GATGGTGTATGGRYCAATCACGACAATTCC 3'

R represents a mixture of the two bases: A and G used in the synthesis of the mutagenic oligonucleotide and Y represents a mixture of the two bases: C and T used in the synthesis of the mutagenic oligonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 168 in the mature amylase.

Construction of the variant: delta(D183-G184) + Q169N is carried out in a similar way except that mutagenic primer DA15 is used.

primer DA15 (SEQ ID NO:28):

5' GGTGTATGGGATAACTCACGACAATTCC 3'

Construction of the variant: delta(D183-G184) + Q169L is carried out in a similar way except that mutagenic primer DA16 is used.

primer DA16 (SEQ ID NO:29):

5' GGTGTATGGGATCTCTCACGACAATTCC 3'

Construction of the variant: delta(D183-G184) + Q172N is carried out in a similar way except that mutagenic primer DA17 is used.

primer DA17 (SEQ ID NO:30):

5′ GGGATCAATCACGAAATTTCCAAAATCGTATC 3′

Construction of the variant: delta(D183-G184) + Q172L is carried out in a similar way except that mutagenic primer DA18 is used.

primer DA18 (SEQ ID NO:31):

5′ GGGATCAATCACGACTCTTCCAAAATCGTATC 3′

Construction of the variant: delta(D183-G184) + L201I is carried out in a similar way except that mutagenic primer DA06 is used.

primer DA06 (SEQ ID NO:32):

5′ GGAAATTATGATTATATCATGTATGCAGATGTAG 3′

Construction of the variant: delta(D183-G184) + K269S is carried out in a similar way except that mutagenic primer DA09 is used.

primer DA09 (SEQ ID NO:33):

5′ GCTGAATTTTGGTCGAATGATTTAGGTGCC 3′

Construction of the variant: delta(D183-G184) + K269Q is carried out in a similar way except that mutagenic primer DA11 is used.

primer DA11 (SEQ ID NO:34):

5′ GCTGAATTTTGGTCGAATGATTTAGGTGCC 3′

Construction of the variant: delta(D183-G184) + N270Y is carried out in a similar way except that mutagenic primer DA21 is used.

primer DA21 (SEQ ID NO:35):

5′ GAATTTTGGAAGTACGATTTAGGTCGG 3′

Construction of the variants: delta(D183-G184) + L272A, delta(D183-G184) + L272I, delta(D183-G184) + L272V, delta(D183-G184) + L272T is carried out in a similar way except that mutagenic primer DA12 is used.

primer DA12 (SEQ ID NO:36):

5′ GGAAAAACGATRYCGGTGCCTTGGAGAAC 3′

R represents a mixture of the two bases: A and G used in the synthesis of the mutagenic oligonucleotide and Y represents a mixture of the two bases: C and T used in the synthesis of the mutagenic oligonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 272 in the mature amylase.

Construction of the variants: delta(D183-G184) + L275A, delta(D183-G184) + L275I, delta(D183-G184) + L275V, delta(D183-G184) + L275T is carried out in a similar way except that mutagenic primer DA13 is used.

primer DA13 (SEQ ID NO:37):

5' GATTTAGGTGCCTRYCAGAACTATTTA 3'

R represents a mixture of the two bases: A and G used in the synthesis of the mutagenic oligonucleotide and Y represents a mixture of the two bases: C and T used in the synthesis of the mutagenic oligonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 275 in the mature amylase.

Construction of the variant: delta(D183-G184) + Y295E is carried out in a similar way except that mutagenic primer DA08 is used.

primer DA08 (SEQ ID NO:38):

5' CCCCCTTCATGAGAATCTTTATAACG 3'

Construction of delta(D183-G184) + K446Q by the mega-primer method as described by Sarkar and Sommer,1990 (BioTechniques 8: 404-407):

Gene specific primer DA04, annealing 214-231 bp downstream relative to the STOP-codon and mutagenic primer DA10 were used to amplify by PCR an approximately 350 bp DNA fragment from a pTVB112-like plasmid (with the delta(D183-G184) mutations in the gene encoding the amylase depicted in SEQ ID NO: 2).

The resulting DNA fragment is used as a mega-primer together with primer DA05 in a PCR on pTVB112 like plasmid (with mutations delta(D183-G184)). The app. 460 bp product of the second PCR is digested with restriction endonucleases SnaB I and Not I and cloned into pTVB112 like plasmid (delta(D183-G184)) digested with the same enzymes.

primer DA04 (SEQ ID NO:39):

5' GAATCCGAACCTCATTACACATTCG 3'

primer DA05 (SEQ ID NO:40):

5' CGGATGGACTCGAGAAGGAAATACCACG 3'

primer DA10 (SEQ ID NO:41):

5' CGTAGGGCAAAATCAGGCCGGTCAAGTTTGG 3'

Construction of the variants: delta(D183-G184) + K458R is

carried out in a similar way except that mutagenic primer DA22 is used.

primer DA22 (SEQ ID NO:42):

5' CATAACTGGAAATCGCCCGGGAACAGTTACG 3'

Construction of the variants: delta(D183-G184) + P459S and delta(D183-G184) + P459T is carried out in a similar way except that mutagenic primer DA19 is used.

primer DA19 (SEQ ID NO:43):

5' CTGGAAATAAAWCCGGAACAGTTACG 3'

W represents a mixture of the two bases: A and T used in the synthesis of the mutagenic oligonucleotide.

Sequencing of transformants identifies the correct codon for amino acid position 459 in the mature amylase.

Construction of the variants: delta(D183-G184) + T461P is carried out in a similar way except that mutagenic primer DA23 is used.

primer DA23 (SEQ ID NO:44):

5' GGAAATAAACCAGGACCCGTTACGATCAATGC 3'

Construction of the variant: delta(D183-G184) + K142R is carried out in a similar way except that mutagenic primer DA32 is used.

Primer DA32 (SEQ ID NO: 45):

5' GAGGCTTGGACTAGGTTTGATTTTCCAG 3'

Construction of the variant: delta(D183-G184) + K269R is carried out in a similar way except that mutagenic primer DA31 is used.

Primer DA31 (SEQ ID NO: 46):

5' GCTGAATTTTGGCGCAATGATTTAGGTGCC 3'

Example 6

Construction of site-directed α-amylase variants in the parent Termamyl α-amylase (SEQ ID NO: 4)

The amyL gene, encoding the Termamyl α-amylase is located in plasmid pDN1528 described in WO 95/10603 (Novo Nordisk). Variants with substitutions N265R and N265D, respectively, of said parent α-amylase are constructed by methods described in WO 97/41213 or by the "megaprimer" approach described above.

Mutagenic oligonucleotides are:

Primer b11 for the N265R substitution:

5' PCC AGC GCG CCT AGG TCA CGC TGC CAA TAT TCA G (SEQ ID NO: 56)

Primer b12 for the N265D substitution:

5' PCC AGC GCG CCT AGG TCA TCC TGC CAA TAT TCA G (SEQ ID NO: 57)

P represents a phosphate group.

Example 7

Determination of pH stability at alkaline pH of variants of the parent α-Amylase having the amino acid sequence shown in SEQ ID NO:2.

In this serie of analysis purified enzyme samples were used. The measurements were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5. The solutions were incubated at 75°C.

After incubation for 20 and 30 min the residual activity was measured using the PNP-G7 assay (described in the "Materials and Methods" section above). The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 75°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	Residual activity after 20 min	Residual activity after 30 min
Δ(D183-G184)+M323L	56 %	44 %
Δ(D183-G184)+M323L+R181S	67 %	55 %
Δ(D183-G184)+M323L+A186T	62 %	50 %

In an other series of analysis culture supernatants were used. The measurements were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5.

The solutions were incubated at 80°C.

After incubation for 30 minutes the residual activity was measured using the Phadebas assay (described in the "Materials and Method" secion above. The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 80°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	Residual activity after 30 min
Δ(D183-G184)	4 %
Δ(D183-G184)+P459T	25 %
Δ(D183-G184)+K458R	31 %
Δ(D183-G184)+K311R	10 %

Example 8

Determination of calcium stability at alkaline pH of variants of the parent α-Amylase having the amino acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 4.

A: Calcium stability of variants of the sequence in SEQ ID NO:1

The measurement were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5 to which polyphosphate was added (at time t=0) to give a final concentration of 2400 ppm. The solutions were incubated at 50°C.

After incubation for 20 and 30 minutes the residual activity was measured using the PNP-G7 assay (described above). The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 50°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 1) and for

the variants in question.

Variant	Residual activity after 20 min	Residual activity after 30 min
Δ(T183-G184)	32 %	19 %
Δ(T183-G184)+ A186T	36 %	23 %
Δ(T183-G184)+K185R+A186T	45 %	29 %
Δ(T183-G184)+A186I	35 %	20 %
Δ(T183-G184)+N195F	44 %	n.d.

n.d.= Not determinated

B: Calcium stability of variants of the sequence in SEQ ID NO:2

In this series of analysis purified samples of enzymes were used. The measurement were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5 to which polyphosphate was added (at time t=0) to give a final concentration of 2400 ppm. The solutions were incubated at 50°C. After incubation for 20 and 30 minutes the residual activity was measured using the PNP-G7 assay (described above). The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 50°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	Residual activity after 20 min	Residual activity after 30 min
Δ(D183-G184)+M323L	21 %	13 %
Δ(D183-G184)+M323L+R181S	32 %	19 %
Δ(D183-G184)+M323L+A186T	28 %	17 %
Δ(D183-G184)+M323L+A186R	30 %	18 %

Variant	Residual activity after 20 min	Residual activity after 30 min

Δ(D183-G184)	30%	20%
Δ(D183-G184)+N195F	55%	44%

In this serie of analysis culture supernatants were used. The measurement were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5 to which polyphosphate was added (at time t=0) to give a final concentration of 2400 ppm. The solutions were incubated at 50°C.

After incubation for 30 minutes the residual activity was measured using the Phadebas assay as described above. The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 50°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	Residual activity after 30 min
Δ(D183-G184)	0 %
Δ(D183-G184)+P459T	19 %
Δ(D183-G184)+K458R	18 %
Δ(D183-G184)+T461P	13 %
Δ(D183-G184)+E346Q+K385R	4 %

C: Calcium stability of variants of the sequence in SEQ ID NO:4

The measurement were made using solutions of the respective variants in 100 mM CAPS buffer adjusted to pH 10.5 to which polyphosphate was added (at time t=0) to give a final concentration of 2400 ppm. The solutions were incubated at 60°C for 20 minutes.

After incubation for 20 minutes the residual activity was measured using the PNP-G7 assay (described above). The residual activity in the samples was measured using Britton Robinson buffer pH 7.3. The decline in residual activity was measured

relative to a corresponding reference solution of the same enzyme at 0 minutes, which has not been incubated at high pH and 60°C.

The percentage of the initial activity as a function is shown in the table below for the parent enzyme (SEQ ID NO: 4) and for the variants in question.

Variant	Residual activity after 20 min
Termamyl (SEQ ID NO: 4)	17 %
N265R	28 %
N265D	25 %

Example 9:

Activity measurement at medium temperature of α-Amylases having the amino acid sequence shown in SEQ ID NO: 1.

A: α-Amylase activity of variants of the sequence in SEQ ID NO:1

The measurement were made using solutions of the respective variants in 50 mM Britton Robinson buffer adjusted to pH 7.3 and using the Phadebas assay described above. The activity in the samples was measured at 37°C using 50 mM Britton Robinson buffer pH 7.3 and at 25°C using 50 mM CAPS buffer pH 10.5.

The temperature dependent activity and the percentage of the activity at 25°C relative to the activity at 37°C are shown in the table below for the parent enzyme (SEQ ID NO: 1) and for the variants in question.

Variant	NU/mg 25°C	NU/mg 37°C	NU(25°C) / NU(37°C)
SP690	1440	35000	4.1 %
Δ(T183-G184)	2900	40000	7.3 %
Δ(T183-G184)+K269S	1860	12000	15.5 %
Δ(Q174)	3830	38000	7.9 %

Another measurement was made using solutions of the

respective variants in 50 mM Britton Robinson buffer adjusted to pH 7.3 and using the Phadebas assay described above. The activity in the samples was measured at 37°C and 50°C using 50 mM Britton Robinson buffer pH 7.3.

The temperature dependent activity and the percentage of the activity at 37°C relative to the activity at 50°C is shown in the table below for the parent enzyme (SEQ ID NO: 1) and for the variants in question.

Variant	NU/mg 37°C	NU/mg 50°C	NU(37°C) / NU(50°C)
SP690 (seq ID NO: 1)	13090	21669	60 %
K269Q	7804	10063	78 %

B: α-Amylase activity of variants of the sequence in SEQ ID NO:2

The measurement were made using solutions of the respective variants in 50 mM Britton Robinson buffer adjusted to pH 7.3 and using the Phadebas assay described above. The activity in the samples was measured at both 25°C and 37°C using 50 mM Britton Robinson buffer pH 7.3.

The temperature dependent activity and the percentage of the activity at 25°C relative to the activity at 37°C is shown in the table below for the parent enzyme (SEQ ID NO: 2) and for the variants in question.

Variant	NU/mg 25°C	NU/mg 37°C	NU(25°C) / NU(37°C)
Δ(D183-G184)+M323L	3049	10202	30 %
Δ(D183-G184)+M323L+R181S	18695	36436	51 %

C: α-Amylase activity of variants of the sequence in SEQ ID NO:4

The measurement were made using solutions of the respective variants in 50 mM Britton Robinson buffer adjusted to pH 7.3 and using the Phadebas assay described above. The activity in

the samples was measured at both 37°C using 50 mM Britton Robinson buffer pH 7.3 and at 60°C using 50 mM CAPS buffer pH 10.5.

The temperature dependent activity and the percentage of the activity at 37°C relative to the activity at 60°C is shown in the table below for the parent enzyme (SEQ ID NO: 4) and for the variants in question.

Variant	NU/mg 37°C	NU/mg 60°C	NU(37°C) / NU(60°C)
Termamyl	7400	4350	170 %
Q264S	10000	4650	215 %

Example 10

Construction of variants of parent hybrid BAN:1-300/Termamyl:301-483 α-amylase

Plasmid pTVB191 contains the gene encoding hybrid α-amylase BAN:1-300/Termamyl:301-483 as well as an origin of replication functional in *Bacillus subtilis* and the *cat* gene conferring chloramphenicol resistance.

Variant BM4 (F290E) was constructed using the megaprimer approach (Sarkar and Sommer, 1990) with plasmid pTVB191 as template.

Primer p1 (SEQ ID NO: 52) and mutagenic oligonucleotide bm4 (SEQ ID NO: 47) were used to amplify a 444 bp fragment with polymerase chain reaction (PCR) under standard conditions. This fragment was purified from an agarose gel and used as 'Megaprimer' in a second PCR with primer p2 (SEQ ID NO: 53) resulting in a 531 bp fragment. This fragment was digested with restriction endonucleases HinDIII and Tth111I. The 389 bp fragment produced by this was ligated into plasmid pTVB191 that had been cleaved with the same two enzymes. The resulting plasmid was transformed into *B. subtilis* SHA273. Chloramphenicol resistant clones were selected by growing the transformants on plates containing chloramphenicol as well as insoluble starch. Clones expressing an active α-amylase were isolated by selecting cloncs that formed halos after staining the plates with iodine vapour. The identity of the introduced mutations was confirmed by DNA sequencing.

Variants BM5(F290K), BM6(F290A), BM8(Q360E) and BM11(N102D) were constructed in a similar way. Details of their construction are given below.

Variant: BM5(F290K)
mutagenic oligonucleotide: bm5 (SEQ ID NO: 48)
Primer (1st PCR): p1 (SEQ ID NO: 52)
Size of resulting fragment: 444 bp
Primer (2nd PCR): p2 (SEQ ID NO: 53)
Restriction endonucleases: HinDIII, Tth111I
Size of cleaved fragment: 389 bp

Variant: BM6(F290A)
mutagenic oligonucleotide: bm6 (SEQ ID NO: 49)
Primer (1st PCR): p1 (SEQ ID NO: 52)
Size of resulting fragment: 444 bp
Primer (2nd PCR): p2 (SEQ ID NO: 53)
Restriction endonucleases: HinDIII, Tth111I
Size of cleaved fragment: 389 bp

Variant: BM8(Q360E)
mutagenic oligonucleotide: bm8 (SEQ ID NO: 50)
Primer (1st PCR): p1 (SEQ ID NO: 52)
Size of resulting fragment: 230 bp
Primer (2nd PCR): p2 (SEQ ID NO: 53)
Restriction endonucleases: HinDIII, Tth111I
Size of cleaved fragment: 389 bp

Variant: BM11(N102D)
mutagenic oligonucleotide: bm11 (SEQ ID NO: 51)
Primer (1st PCR): p3 (SEQ ID NO: 54)
Size of resulting fragment: 577
Primer (2nd PCR): p4 (SEQ ID NO: 55)
Restriction endonucleases: HinDIII, PvuI
Size of cleaved fragment: 576

Mutagenic oligonucleotides:
bm4 (SEQ ID NO: 47): F290E
primer 5' GTG TTT GAC GTC CCG CTT CAT GAG AAT TTA CAG G
bm5 (SEQ ID NO: 48): F290K
primer 5' GTG TTT GAC GTC CCG CTT CAT AAG AAT TTA CAG G
bm6 (SEQ ID NO: 49): F290A
primer 5' GTG TTT GAC GTC CCG CTT CAT GCC AAT TTA CAG G
bm8 (SEQ ID NO: 50): Q360E
primer 5' AGG GAA TCC GGA TAC CCT GAG GTT TTC TAC GG
bm11 (SEQ ID NO: 51): N102D
primer 5' GAT GTG GTT TTG GAT CAT AAG GCC GGC GCT GAT G

Other primers:

p1: 5' CT TTA TTA ATG CGG CCA AAC C (SEQ ID NO: 52)

p2: 5' G GAA AAG AAA TGT TTA CGG TTG CG (SEQ ID NO: 53)

p3: 5' G AAA TGA AGC GGA ACA TCA AAC ACG (SEQ ID NO: 54)

p4: 5' GTA TGA TTT AGG AGA ATT CC (SEQ ID NO: 55)

Example 11

α-Amylase activity at alkaline pH of variants of parent BAN:1-300/Termamyl:301-483 hybrid α-amylase.

The measurements were made using solutions for the respective enzymes and utilizing the Phadebas assay (described above). The activity was measured after incubating for 15 minutes at 30°C in 50 mM Britton-Robinson buffer adjusted to the indicated pH by NaOH.

NU/mg enzyme

pH	wt	Q360E	F290A	F290K	F290E	N102D
8.0	5300	7800	8300	4200	6600	6200
9.0	1600	2700	3400	2100	1900	1900

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CLAIMS

1. A variant of a parent Termamyl-like α-amylase, which variant has α-amylase activity, said variant comprises one or more mutations corresponding to the following mutations in the amino acid sequence shown in SEQ ID NO: 2:
T141, K142, F143, D144, F145, P146, G147, R148, G149,
Q174, R181, G182, D183, G184, K185, A186, W189, S193, N195
H107, K108, G109,D166, W167, D168, Q169, S170, R171, Q172, F173,
F267, W268, K269, N270, D271, L272, G273, A274, L275, K311,
E346, K385, G456, N457, K458,P459, G460, T461, V462, T463.

2. The variant according to claim 1, which variant has one or more of the following substitutions or deletions:
T141A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
K142A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
F143A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
D144A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F145A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
P146A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G147A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
R148A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G149A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
R181*,A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G182*,A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D183*,A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G184*,A,R,D,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
K185A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
A186D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W189A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
S193A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
H107A,D,R,N,C,E,Q,G,I,L,K,M,F,P,S,T,W,Y,V;
K108A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G109A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D166A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W167A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
D168A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

Q169A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S170A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
R171A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q172A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F173A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
Q174*,A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F267A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
W268A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
K269A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N270A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D271A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L272A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
G273A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
A274D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L275A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
K311A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
E346A,D,R,N,C,Q,G,H,I,K,L,M,F,P,S,T,W,Y,V;
K385A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G456A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
N457A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K458A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G460A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
T461A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
V462A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
T463A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V.

3. The variant according to claim 2, wherein the variant has one or more of the following substitutions or deletions:
K142R; S193P; N195F; K269R,Q; N270Y,R,D; K311R; E346Q; K385R; K458R; P459T; T461P; Q174*; R181Q,N,S; G182T,S,N; D183*; G184*; K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V; A186T,S,N,I,V,R; W189T,S,N,Q.

4. The variant according to claims 1-3, wherein the variant has a deletion in position D183 + G184, and further one or more of the following substitutions or deletions: K142R; S193P; N195F; K269R,Q; N270Y,R,D; K311R; E346Q; K385R; K458R; P459T; T461P;

Q174*; R181Q,N,S; G182T,S,N, 183*; G184*;
K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V; A186T,S,N,I,V,R;
W189T,S,N

5. The variant according to any of claims 1-4, wherein the variants exhibits an alteration in at least one of the following properties relative to the parent α-amylase:
i) improved pH stability at a pH from 8 to 10.5; and/or
ii) improved Ca^{2+} stability at pH 8 to 10.5, and/or
iii) increased specific activity at temperatures from 10 to 60°C, preferably 20-50°C, especially 30-40°C.

6. The variant according to any of claims 1-5, exhibiting improved stability at pH 8 to 10.5, having mutations in one or more of the position(s) corresponding to the following positions (using SEQ ID NO: 2 numbering): T141, K142, F143, D144, F145, P146, G147, R148, G149, R181, A186, S193, N195, K269, N270, K311, K458, P459, T461.

7. The variant according to claim 6, which variant has one or more of the following substitutions:
T141A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
K142A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
F143A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
D144A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F145A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
P146A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G147A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
R148A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G149A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
K181A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
A186D,R,N,C,E,Q,G,H,I,L,P,K,M,F,S,T,W,Y,V;
S193A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K269A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N270A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K311A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;

K458A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
T461A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V.

8. The variant according to claim 7, wherein the variant has one or more of the following substitutions: K142R, R181S, A186T, S193P, N195F, K269R, N270Y, K311R, K458R, P459T and T461P.

9. The variant according to claims 1-5, exhibiting improved Ca^{2+} stability at pH 8 to 10.5, having mutations in one or more of the following positions (using the SEQ ID NO: 2 numbering): R181, G182, D183, G184, K185, A186, W189, N195, N270, E346, K385, K458, P459.

10. The variant according to claim 9, which variant has one or more of the following substitutions or deletions:
R181*,A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G182*,A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D183*,A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
G184*,A,R,D,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
K185A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
A186D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W189A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
N270A,R,D,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
E346A,R,D,N,C,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K385A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
K458A,R,D,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,R,D,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V.

11.The variant according to claim 10, wherein the variant has one or more of the following substitutions or deletions:
R181Q,N; G182T,S,N; D183*; G184*;
K185A,R,D,C,E,Q,G,H,I,L,M,N,F,P,S,T,W,Y,V; A186T,S,N,I,V;
W189T,S,N,Q; N195F; N270R,D; E346Q; K385R; K458R; P459T.

12. A variant according to claims 1-11, wherein the parent Termamyl-like α-amylase is selected from:

the *Bacillus* strain NCIB 12512 α-amylase having the sequence shown in SEQ ID NO: 1;

the *B. amyloliquefaciens* α-amylase having the sequence shown in SEQ ID NO: 5;

the *B. licheniformis* α-amylase having the sequence shown in SEQ ID NO: 4.

13. The variant according to claims 1-5, exhibiting increased specific activity at a temperatures from 10 to 60°C, preferably 20-50°C, especially 30-40°C, having mutation(s) in one or more of the following positions (using the SEQ ID NO: 2 numbering): H107, K108, G109, D166, W167, D168, Q169, S170, R171, Q172, F173, Q174, D183, G184, N195, F267, W268, K269,N270, D271, L272, G273, A274, L275, G456, N457, K458, P459, G460, T461, V462, T463.

14. The variant according to claim 13, which variant has one or more of the following substitutions:

H107A,D,R,N,C,E,Q,G,I,L,K,M,F,P,S,T,W,Y,V;
K108A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
G109A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
D166A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
W167A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
D168A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q169A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
S170A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,T,W,Y,V;
R171A,D,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
Q172A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F173A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
Q174*,A,D,R,N,C,E,G,H,I,L,K,M,F,P,S,T,W,Y,V;
D183*,A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
G184*,A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
N195A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
F267A,D,R,N,C,E,Q,G,H,I,L,K,M,P,S,T,W,Y,V;
W268A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,Y,V;
K269A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
N270A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;

D271A,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L272A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
G273A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
A274D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
L275A,D,R,N,C,E,Q,G,H,I,K,M,F,P,S,T,W,Y,V;
G456A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
N457A,D,R,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y,V;
K458A,D,R,N,C,E,Q,G,H,I,L,M,F,P,S,T,W,Y,V;
P459A,D,R,N,C,E,Q,G,H,I,L,K,M,F,S,T,W,Y,V;
G460A,D,R,N,C,E,Q,H,I,L,K,M,F,P,S,T,W,Y,V;
T461A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V;
V462A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,T,W,Y;
T463A,D,R,N,C,E,Q,G,H,I,L,K,M,F,P,S,W,Y,V.

15. The variant according to claim 14, wherein the variant has one or more of the following substitutions or deletions:
Q174*, D183*, G184*, N195F, K269S.

16. The variant according to claims 13-15, wherein the parent Termamyl-like α-amylase is the *B. licheniformis* α-amylase having the sequence shown in SEQ ID NO: 4.

17. A DNA construct comprising a DNA sequence encoding an α-amylase variant according to any one of claims 1-16.

18. A recombinant expression vector which carries a DNA construct according to claim 17.

19. A cell which is transformed with a DNA construct according to claim 17 or a vector according to claim 18.

20. A cell according to claim 19, which is a microorganism.

21. A cell according to claim 20, which is a bacterium or a fungus.

22. The cell according to claim 21, which is a Gram positive bacterium such as *Bacillus subtilis*, *Bacillus licheniformis*,

Bacillus lentus, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus* or *Bacillus thuringiensis*.

23. Use of an α-amylase variant according to any one of claims 1-16 for washing and/or dishwashing.

24. A detergent additive comprising an α-amylase variant according to any one of claims 1-16, optionally in the form of a non-dusting granulate, stabilized liquid or protected enzyme.

25. A detergent additive according to claim 24 which contains 0.02-200 mg of enzyme protein/g of the additive.

26. A detergent additive according to claims 24 or 25, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

27. A detergent composition comprising an α-amylase variant according to any of claims 1-16.

28. A detergent composition according to claim 27 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

29. A manual or automatic dishwashing detergent composition comprising an α-amylase variant according to any one of claims 1-16.

30. A dishwashing detergent composition according to claim 29 which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic enzyme and/or a cellulase.

31. A manual or automatic laundry washing composition comprising

an α-amylase variant according to any one of claims 1-16.

32. A laundry washing composition according to claim 31, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, an amylolytic enzyme and/or a cellulase.

33. Method for providing α-amylases with
1) altered pH optimum, and/or
2) altered temperature optimum, and/or
3) improved stability,
comprising the following steps:
i) identifying (a) target position(s) and/or region(s) for mutation of the α-amylase by comparing the molecular dynamics of two or more α-amylase's 3D structures having substantially different pH, temperature and/or stability profiles,
ii) substituting, adding and/or deleting one or more amino acids in the identified position(s) and/or region(s).

34. The method according to claim 33, wherein a medium temperature α-amylase is compared with a high temperature α-amylase.

35. The method according to claim 33, wherein a low temperature α-amylase is compared with a medium or high temperature α-amylase.

36. The method according to claims 33-35, wherein the α-amylases are at least 70%, preferably 80%, up to 90%, such as up to 95%, especially 95% homologous.

37. The method according to claim 36, wherein the α-amylases compared are Termamyl-like α-amylases.

38. The method according to claim 28, wherein the α-amylases compared are any of the α-amylases shown in SEQ ID NO: 1 to SEQ ID NO: 8.

```
   1                                                  50
1  HHNGTNGTMM QYFEWHLPND GNHWNRLRDD ASNLRNRGIT AIWIPPAWKG
2  ..NGTNGTMM QYFEWYLPND GNHWNRLRSD ASNLKDKGIS AVWIPPAWKG
3  HHNGTNGTMM QYFEWYLPND GNHWNRLRDD AANLKSKGIT AVWIPPAWKG
4  ....VNGTLM QYFEWYTPND GQHWKRLQND AEHLSDIGIT AVWIPPAYKG
5  ..ANLNGTLM QYFEWYMPND GQHWRRLQND SAYLAEHGIT AVWIPPAYKG
6  .AAPFNGTMM QYFEWYLPDD GTLWTKVANE ANNLSSLGIT ALWLPPAYKG

   51                                                 100
1  TSQNDVGYGA YDLYDLGEFN QKGTVRTKYG TRSQLESAIH ALKNNGVQVY
2  ASQNDVGYGA YDLYDLGEFN QKGTIRTKYG TRNQLQAAVN ALKSNGIQVY
3  TSQNDVGYGA YDLYDLGEFN QKGTVRTKYG TRNQLQAAVT SLKNNGIQVY
4  LSQSDNGYGP YDLYDLGEFQ QKGTVRTKYG TKSELQDAIG SLHSRNVQVY
5  TSQADVGYGA YDLYDLGEFH QKGTVRTKYG TKGELQSAIK SLHSRDINVY
6  TSRSDVGYGV YDLYDLGEFN QKGTVRTKYG TKAQYLQAIQ AAHAAGMQVY

   101                                                150
1  GDVVMNHKGG ADATENVLAV EVNPNNRNQE ISGDYTIEAW TKFDFPGRGN
2  GDVVMNHKGG ADATEMVRAV EVNPNNRNQE VSGEYTIEAW TKFDFPGRGN
3  GDVVMNHKGG ADGTEIVNAV EVNRSNRNQE TSGEYAIEAW TKFDFPGRGN
4  GDVVLNHKAG ADATEDVTAV EVNPANRNQE TSEEYQIKAW TDFRFPGRGN
5  GDVVINHKGG ADATEDVTAV EVDPADRNRV ISGEHLIKAW THFHFPGRGS
6  ADVVFDHKGG ADGTEWVDAV EVNPSDRNQE ISGTYQIQAW TKFDFPGRGN
```

Fig. 1

```
     151                                                  200
1    TYSDFKWRWY HFDGVDWDQS RQFQNRIYKF RGDGKAWDWE VDSENGNYDY
2    THSNFKWRWY HFDGVDWDQS RKLNNRIYKF RGDGKGWDWE VDTENGNYDY
3    NHSSFKWRWY HFDGTDWDQS RQLQNKIYKF RGTGKAWDWE VDTENGNYDY
4    TYSDFKWHWY HFDGADWDES RKI.SRIFKF RGEGKAWDWE VSSENGNYDY
5    TYSDFKWHWY HFDGTDWDES RKL.NRIYKF ..QGKAWDWE VSNENGNYDY
6    TYSSFKWRWY HFDGVDWDES RKL.SRIYKF RGIGKAWDWE VDTENGNYDY

     201                                                  250
1    LMYADVDMDH PEVVNELRRW GEWYTNTLNL DGFRIDAVKH IKYSFTRDWL
2    LMYADIDMDH PEVVNELRNW GVWYTNTLGL DGFRIDAVKH IKYSFTRDWS
3    LMYADVDMDH PEVIHELRNW GVWYTNTLNL DGFRIDAVKH IKYSFTRDWL
4    LMYADVDYDH PDVVAETKKW GIWYANELSL DGFRIDAAKH IKFSFLRDWV
5    LMYADIDYDH PDVAAEIKRW GTWYANELQL DGFRLDAVKH IKFSFLRDWV
6    LMYADLDMDH PEVVTELKNW GKWYVNTTNI DGFRLDAVKH IKFSFFPDWL

     251                                                  300
1    THVRNATGKE MFAVAEFWKN DLGALENYLN KTNWNHSVFD VPLHYNLYNA
2    IHVRSATGKN MFAVAEFWKN DLGAIENYLN KTNWNHSVFD VPLHYNFYNA
3    THVRNTTGKP MFAVAEFWKN DLGAIENYLN KTSWNHSAFD VPLHYNLYNA
4    QAVRQATGKE MFTVAEYWQN NAGKLENYLN KTSFNQSVFD VPLHFNLQAA
5    NHVREKTGKE MFTVAEYWQN DLGALENYLN KTNFNHSVFD VPLHYQFHAA
6    SYVRSQTGKP LFTVGEYWSY DINKLHNYIT KTDGTMSLFD APLHNKFYTA
```

Fig. 1 (continued)

```
   301                                                350
1  SNSGGNYDMA KLLNGTVVQK HPMHAVTFVD NHDSQPGESL ESFVQEWFKP
2  SKSGGNYDMR QIFNGTVVQR HPMHAVTFVD NHDSQPEEAL ESFVEEWFKP
3  SNSGGYYDMR NILNGSVVQK HPTHAVTFVD NHDSQPGEAL ESFVQQWFKP
4  SSQGGGYDMR RLLDGTVVSR HPEKAVTFVE NHDTQPGQSL ESTVQTWFKP
5  STQGGGYDMR KLLNGTVVSK HPLKSVTFVD NHDTQPGQSL ESTVQTWFKP
6  SKSGGAFDMR TLMTNTLMKD QPTLAVTFVD NHDTEPGQAL QSWVDPWFKP

   351                                                400
1  LAYALILTRE QGYPSVFYGD YYGIPTHS.. .VPAMKAKID PILEARQNFA
2  LAYALTLTRE QGYPSVFYGD YYGIPTHG.. .VPAMKSKID PILEARQKYA
3  LAYALVLTRE QGYPSVFYGD YYGIPTHG.. .VPAMKSKID PLLQARQTFA
4  LAYAFILTRE SGYPQVFYGD MYGTKGTSPK EIPSLKDNIE PILKARKEYA
5  LAYAFILTRE SGYPQVFYGD MYGTKGDSQR EIPALKHKIE PILKARKQYA
6  LAYAFILTRQ EGYPCVFYGD YYGIPQYN.. .IPSLKSKID PLLIARRDYA

   401                                                450
1  YGTQHDYFDH HNIIGWTREG NTTHPNSGLA TIMSDGPGGE KWMYVGQNKA
2  YGRQN..... .......... .......... .......... ..........
3  YGTQHDYFDH HDIIGWTREG NSSHPNSGLA TIMSDGPGGN KWMYVGKNKA
4  YGPQHDYIDH PDVIGWTREG DSSAAKSGLA ALITDGPGGS KRMYAGLKNA
5  YGAQHDYFDH HDIVGWTREG DSSVANSGLA ALITDGPGGA KRMYVGRQNA
6  YGTQHDYLDH SDIIGWTREG GTEKPGSGLA ALITDGPGGS KWMYVGKQHA
```

Fig.1 (continued)

```
                451                                                500
  1 GQVWHDITGN KPGTVTINAD GWANFSVNGG SVSIWVKR.. ..........
  2 .......... .......... .......... .......... ..........
  3 GQVWRDITGN RTGTVTINAD GWGNFSVNGG SVSVWVKQ.. ..........
  4 GETWYDITGN RSDTVKIGSD GWGEFHVNDG SVSIYVQ... ..........
  5 GETWHDITGN RSEPVVINSE GWGEFHVNGG SVSIYVQR.. ..........
  6 GKVFYDLTGN RSDTVTINSD GWGEFKVNGG SVSVWVPRKT TVSTIARPIT

                501        519
  1 .......... .........
  2 .......... .........
  3 .......... .........
  4 .......... .........
  5 .......... .........
  6 TRPWTGEFVR WTEPRLVAW
```

Fig. 1 (continued)

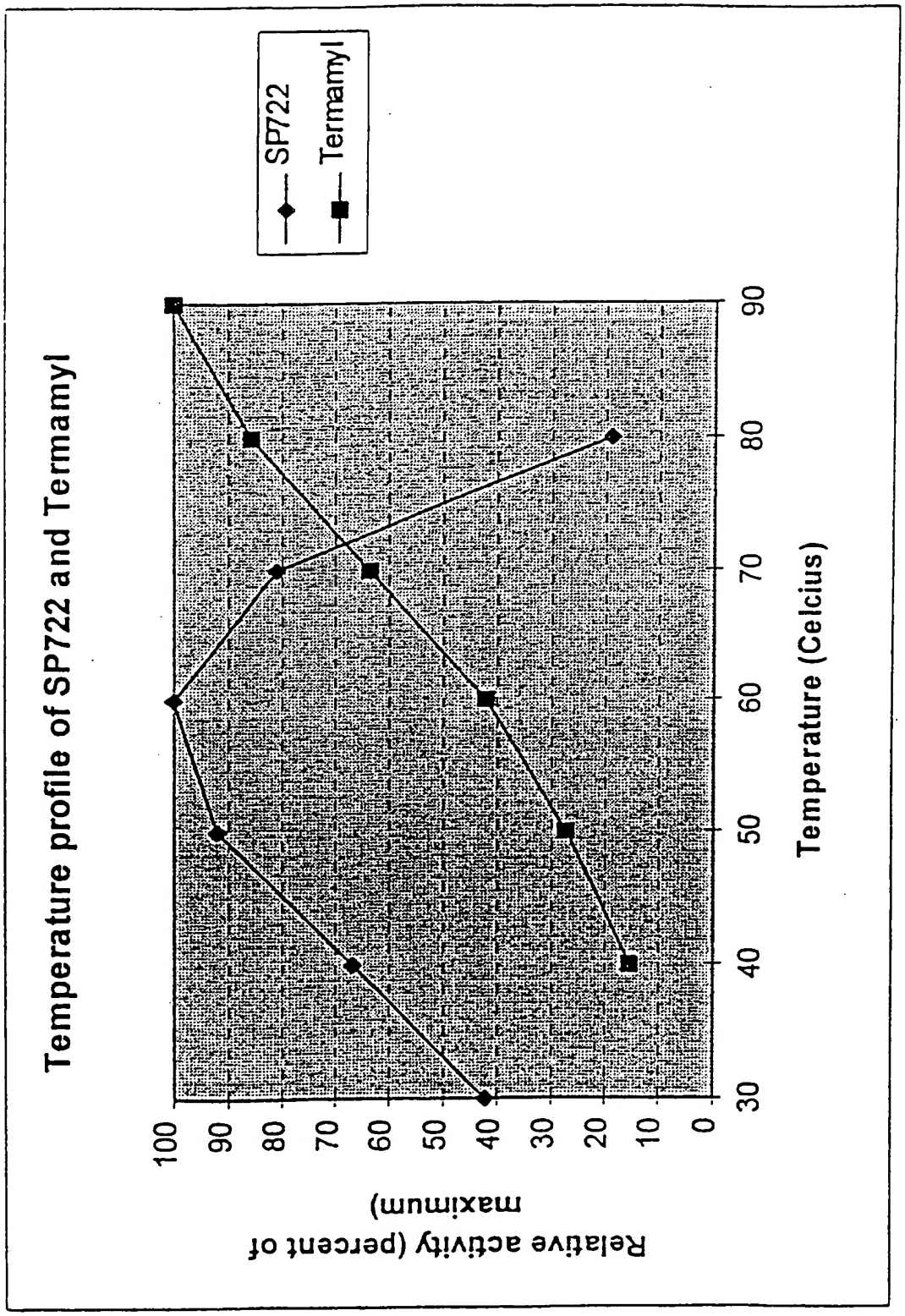

Fig. 2

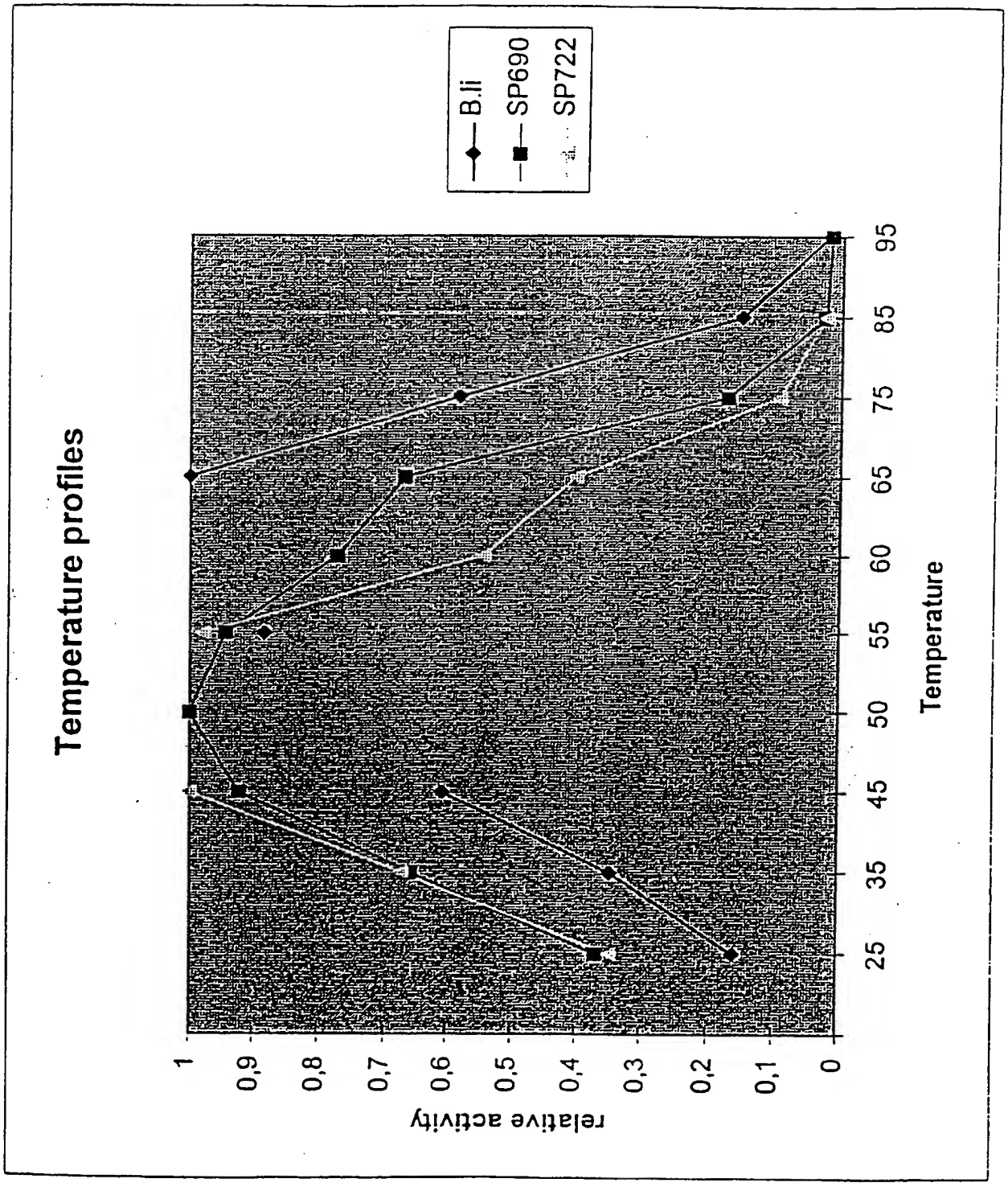

Fig. 3

```
     1                                                    50
   1 MKFVLLLSLI GFCWAQYDPH TSDG.RTAIV HLFEWRWVDI AKECERYLAP
   2 .....LLSLI GFCWAQYDPH TADG.RTAIV HLFEWRWADI AKECERYLAP
   3 .......... .....QYAPQ TQSG.RTDIV HLFEWRWVDI ALECERYLGP
   4 MKFFLLLFTI GFCWAQYSPN TQQG.RTSIV HLFEWRWVDI ALECERYLAP
   5 MKLNKIITTA GLSLGLLLPS IATATPTTFV HLFEWNWQDV AQECEQYLGP

     51                                                  100
   1 KGFGGVQVSP PNENVVVHNP SRPWWERYQP ISYKICTRSG NEDEFRDMVT
   2 KGFGGVQVSP PNENIIINNP SRPWWERYQP ISYKICSRSG NENEFKDMVT
   3 KGFGGVQVSP PNENVVVTNP SRPWWERYQP VSYKLCTRSG NENEFRDMVT
   4 KGFGGVQVSP PNENVAIYNP FRPWWERYQP VSYKLCTRSG NEDEFRNMVT
   5 KGYAAVQVSP PNEHI....T GSQWWTRYQP VSYELQSRGG NRAQFIDMVN

     101                                                 150
   1 RCNNVGVRIY VDAVINHMCG AGNPAGTSST CGSYLNPNNR EFPAVPYSAW
   2 RCNNVGVRIY VDAVINHMCG SGNSAGTHST CGSYFNPNNR EFSAVPYSAW
   3 RCNNVGVRIY VDAVINHMCG SGAAAGTGTT CGSYCNPGNR EFPAVPYSAW
   4 RCNNVGVRIY VDAVINHMCG NAVSAGTSST CGSYFNPGSR DFPAVPYSGW
   5 RCSAAGVDIY VDTLINHM.. .AAGSGTGTA GNSF...GNK SFPI..YSPQ

     151                                                 200
   1 DFNDNKCN.. .GEIDNYNDA YQVRNCRLTG LLDLALEKDY VRTKVADYMN
   2 YFNDNKCN.. .GEINNYNDA NQVRNCRLSG LLDLALDKDY VRTKVADYMN
   3 DFNDGKCKTA SGGIESYNDP YQVRDCQLVG LLDLALEKDY VRSMIADYLN
   4 DFNDGKCKTG SGDIENYNDA TQVRDCRLTG LLDLALEKDY VRSKIAEYMN
   5 DFHES.CTIN NSDYG..NDR YRVQNCELVG LADLDTASNY VQNTIAAYIN
```

Fig. 4

```
                                                                  250
 1 HLIDIGVAGF RLDAAKHMWP RDIKAVLDKL HNLNTKWFSQ GSRPFIFQEV
 2 NLIDIGVAGF RLDAAKHMWP GDIKAVLDKL HNLNTKWFSQ GSRPFIFQEV
 3 KLIDIGVAGF RLDASKHMWP GDIKAVLDKL HNLNTNWFPA GSRPFIFQEV
 4 HLIDIGVAGF RLDASKHMWP GDIKAILDKL HNLNSNWFPA GSKPFIYQEV
 5 DLQAIGVKGF RFDASKHVAA SDIQSLMAKV N.........  .GSPVVFQEV

                                                                  300
 1 IDLGGEAIKG SEYFGNGRVT EFKYGAKLGT VIRKWNGEKM SYLKNWGEGW
 2 IDLGGEAIKG SEYFGNGRVT EFKYGAKLGT VIRKWNGEKM SYLKNWGEGW
 3 IDLGGEAIKS GEYFSNGRVT EFKYGAKLGT VVRKWSGEKM SYLKNWGEGW
 4 IDLGGEPIKS SDYFGNGRVT EFKYGAKLGT VIRKWNGEKM SYLKNWGEGW
 5 IDQGGEAVGA SEYLSTGLVT EFKYSTELGN TFR...NGSL AWLSNFGEGW

                                                                  350
 1 GLVPSDRALV FVDNHDNQRG HGAGGSSILT FWDARMYKMA VGFMLAHPYG
 2 GFVPTDRALV FVDNHDNQRG HGAGGASILT FWDARMYKMA VGFMLAHPYG
 3 GFMPSDRALV FVDNHDNQRG HGAGGSSILT FWDAYRKLVA VGFMLAHPYG
 4 GFVPSDRALV FVDNHDNQRG HGAGGASILT FWDARLYKMA VGFMLAHPYG
 5 GFMPSSSAVV FVDNHDNQRG HGGAG.NVIT FEDGRLYDLA NVFMLAYPYG
```

Fig. 4 (continued)

```
  351                                                400
1 FTRVMSSYRW NRNFQNGKDQ NDWIGPPNNN GVTKEVTINA DTTCGNDWVC
2 FTRVMSSYRR TRNFQNGKDV NDWIGPPNNN GVTKEVTINP DTTCGNDWVC
3 FTRVMSSYRW ARNFVNGEDV NDWIGPPNNN GVIKEVTINA DTTCGNDWVC
4 FTRVMSSYRW PRQFQNGNDV NDWVGPPNNN GVIKEVTINP DTTCGNDWVC
5 YPKVMSSY.. ..DFHGDTDA GGPNVPVHNN GNLE..... ..CFASNWKC

  401                                                450
1 EHRWRQIRNM VAFRNVVNGQ .PFSNWWDNN SNQVAFSRGN RGFIVFNNDD
2 EHRWRQIRNM VAFRNVVNGQ .PFANWWDNG SNQVAFSRGN RGFIVFNNDD
3 EHRWREIRNM VWFRNVVDGE .PFANWWDNG SNQVAFGRGN RGFIVFNNDD
4 EHRWRQIRNM VIFRNVVDGQ .PFTNWYDNG SNQVAFGRGN RGFIVFNNDD
5 EHRWSYIAGG VDFRNNTADN WAVTNWWDNT NNQISFGRGS SGHMAINKED

  451                                                500
1 WALSATLQTG LPAGTYCDVI SGDKVDG..N CTGLRVNVGS DGKAHFSISN
2 WALSSTLQTG LPAGTYCDVI SGDKVNG..N CTGLKVNVGS DGKAHFSISN
3 WQLSSTLQTG LPAGTYCDVI SGDKVGN..S CTGIKVYVSS DGKAQFSISN
4 WSFSLTLQTG LPAGTYCDVI SGDKING..N CTGIKIYVSD DGKAHFSISN
5 STLTATVQTD MASGQYCNVL KGELSADAKS CSGEVITVNS DGTINLNIGA

  501          521
1 SAEDPFIAIH ADSKL.....
2 SAEDPFIAIH ADSKL.....
3 SAEDPFIAIH AESKL.....
4 SAEDPFIAIH AESKL.....
5 WDA...MAIH KNAKLNTSSA S
```

Fig. 4 (continued)

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT:
(A) NAME: NOVO NORDISK A/S
(B) STREET: Novo Alle
(C) CITY: DK-2880 Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 44 44 88 88
(H) TELEFAX: +45 44 49 32 56
(ii) TITLE OF INVENTION: α-amylase mutants
(iii) NUMBER OF SEQUENCES: 46
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 485 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1               5                  10                  15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
            20                  25                  30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
        35                  40                  45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
    50                  55                  60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65                  70                  75                  80

Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
                85                  90                  95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
            100                 105                 110

Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
        115                 120                 125

Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160
```

His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
165 170 175

Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
195 200 205

Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
210 215 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225 230 235 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
245 250 255

Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
260 265 270

Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
275 280 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
290 295 300

Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
305 310 315 320

His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
325 330 335

Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
340 345 350

Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
355 360 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
370 375 380

Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
385 390 395 400

Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
405 410 415

Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
435 440 445

Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480

Val Trp Val Lys Gln
485

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 485 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
20 25 30

Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80

Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
85 90 95

Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110

Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
115 120 125

Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
130 135 140

Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg
165 170 175

Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190

Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
195 200 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr
210 215 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His

225 230 235 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala
245 250 255

Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
260 265 270

Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
275 280 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
290 295 300

Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys
305 310 315 320

His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
325 330 335

Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala
340 345 350

Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
355 360 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala
370 375 380

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr
385 390 395 400

Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
405 410 415

Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly
435 440 445

Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480

Ile Trp Val Lys Arg
485

(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 514 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1               5                  10                  15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
            20                  25                  30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Ala Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85                  90                  95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115                 120                 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
        195                 200                 205

Pro Glu Val Val Thr Glu Leu Lys Ser Trp Gly Lys Trp Tyr Val Asn
    210                 215                 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225                 230                 235                 240

Phe Ser Phe Phe Pro Asp Trp Leu Ser Asp Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Met Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Thr
    290                 295                 300
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Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
        435                 440                 445

Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
    450                 455                 460

Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465                 470                 475                 480

Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Trp Ser Ile Thr Thr
                485                 490                 495

Arg Pro Trp Thr Asp Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
            500                 505                 510

Ala Trp
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 483 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro
1               5                   10                  15

Asn Asp Gly Gln His Trp Arg Arg Leu Gln Asn Asp Ser Ala Tyr Leu
            20                  25                  30

Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly
```

35 40 45

Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
50 55 60

Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65 70 75 80

Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn
85 90 95

Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr
100 105 110

Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val
115 120 125

Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro
130 135 140

Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe
145 150 155 160

Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys
165 170 175

Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn
180 185 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val
195 200 205

Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln
210 215 220

Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe
225 230 235 240

Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
245 250 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn
260 265 270

Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu
275 280 285

His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
290 295 300

Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
305 310 315 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
325 330 335

Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
340 345 350

Thr Arg Glu Ser Gly Pro Val Phe Tyr Gly Asp Met Tyr Gly
355 365

Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
370 375 380

Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
385 390 395 400

Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
405 410 415

Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
420 425 430

Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
435 440 445

Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
450 455 460

Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
465 470 475 480

Val Gln Arg

(2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 480 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp
1 5 10 15

Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu Ser Asp
20 25 30

Ile Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Leu Ser
35 40 45

Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu Tyr Asp Leu Gly Glu
50 55 60

Phe Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ser Glu
65 70 75 80

Leu Gln Asp Ala Ile Gly Ser Leu His Ser Arg Asn Val Gln Val Tyr
85 90 95

Gly Asp Val Val Leu Asn His Lys Ala Gly Ala Asp Ala Thr Glu Asp
100 105 110

```
Val Thr Ala Val Glu Val Asn Pro Ala Asn Arg Asn Gln Glu Thr Ser
        115                 120                 125

Glu Glu Tyr Gln Ile Lys Ala Trp Thr Asp Phe Arg Phe Pro Gly Arg
    130                 135                 140

Gly Asn Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly
145                 150                 155                 160

Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg Ile Phe Lys Phe Arg
                165                 170                 175

Gly Glu Gly Lys Ala Trp Asp Trp Glu Val Ser Ser Glu Asn Gly Asn
            180                 185                 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Tyr Asp His Pro Asp Val
        195                 200                 205

Val Ala Glu Thr Lys Lys Trp Gly Ile Trp Tyr Ala Asn Glu Leu Ser
    210                 215                 220

Leu Asp Gly Phe Arg Ile Asp Ala Ala Lys His Ile Lys Phe Ser Phe
225                 230                 235                 240

Leu Arg Asp Trp Val Gln Ala Val Arg Gln Ala Thr Gly Lys Glu Met
                245                 250                 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala Gly Lys Leu Glu Asn
            260                 265                 270

Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val Phe Asp Val Pro Leu
        275                 280                 285

His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Gly Tyr Asp Met
    290                 295                 300

Arg Arg Leu Leu Asp Gly Thr Val Val Ser Arg His Pro Glu Lys Ala
305                 310                 315                 320

Val Thr Phe Val Glu Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
                325                 330                 335

Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
            340                 345                 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
        355                 360                 365

Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp Asn Ile
    370                 375                 380

Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Pro Gln His
385                 390                 395                 400

Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp Thr Arg Glu Gly Asp
                405                 410                 415

Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
```

420 425 430

Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys Asn Ala Gly Glu Thr
435 440 445

Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Lys Ile Gly Ser
450 455 460

Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser Ile Tyr
465 470 475 480

(2) INFORMATION FOR SEQ ID NO: 6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 485 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Asn Ser Asp Ala Ser
20 25 30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
35 40 45

Lys Gly Ala Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80

Thr Arg Ser Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
85 90 95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110

Ala Thr Glu Met Val Arg Ala Val Glu Val Asn Pro Asn Asn Arg Asn
115 120 125

Gln Glu Val Thr Gly Glu Tyr Thr Ile Glu Ala Trp Thr Arg Phe Asp
130 135 140

Phe Pro Gly Arg Gly Asn Thr His Ser Ser Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Arg Leu Asn Asn Arg
165 170 175

Ile Tyr Lys Phe Arg Gly His Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190

```
Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
        195                 200                 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr
    210                 215                 220

Thr Asn Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Ile Asn His Val Arg Ser Ala
                245                 250                 255

Thr Gly Lys Asn Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
            260                 265                 270

Gly Ala Ile Glu Asn Tyr Leu Gln Lys Thr Asn Trp Asn His Ser Val
        275                 280                 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Lys Ser Gly
    290                 295                 300

Gly Asn Tyr Asp Met Arg Asn Ile Phe Asn Gly Thr Val Val Gln Arg
305                 310                 315                 320

His Pro Ser His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
                325                 330                 335

Glu Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Arg Ser
    370                 375                 380

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Lys Tyr Ala Tyr Gly Lys
385                 390                 395                 400

Gln Asn Asp Tyr Leu Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
                405                 410                 415

Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
            420                 425                 430

Gly Ala Gly Gly Ser Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
        435                 440                 445

Gln Val Trp Ser Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
    450                 455                 460

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465                 470                 475                 480

Ile Trp Val Asn Lys
                485
```

(2) INFORMATION FOR SEQ ID NO: 7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 485 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1               5                  10                  15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
            20                  25                  30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
        35                  40                  45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
    50                  55                  60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65                  70                  75                  80

Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
                85                  90                  95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
            100                 105                 110

Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
        115                 120                 125

Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160

His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
                165                 170                 175

Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
        195                 200                 205

Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr
    210                 215                 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
                245                 250                 255
```

Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
260 265 270

Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
275 280 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
290 295 300

Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
305 310 315 320

His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
325 330 335

Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala
340 345 350

Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
355 360 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
370 375 380

Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr
385 390 395 400

Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
405 410 415

Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly
435 440 445

Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480

Val Trp Val Lys Gln
485

(2) INFORMATION FOR SEQ ID NO: 8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 485 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser

20 25 30

Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80

Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
85 90 95

Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110

Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
115 120 125

Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
130 135 140

Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg
165 170 175

Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
180 185 190

Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
195 200 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr
210 215 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225 230 235 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala
245 250 255

Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
260 265 270

Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
275 280 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
290 295 300

Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys
305 310 315 320

His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
325 330 335

Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala
340 345 350

Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
355 360 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala
370 375 380

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr
385 390 395 400

Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
405 410 415

Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly
435 440 445

Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465 470 475 480

Ile Trp Val Lys Arg
485

(2) INFORMATION FOR SEQ ID NO: 9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1455 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CATCATAATG GAACAAATGG TACTATGATG CAATATTTCG AATGGTATTT GCCAAATGAC 60

GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA 120

GCTGTATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGTAGG TTATGGAGCC 180

TATGATTTAT ATGATCTTGG AGAGTTTAAC CAGAAGGGGA CGGTTCGTAC AAAATATGGA 240

ACACGCAACC AGCTACAGGC TGCGGTGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT 300

GGTGATGTCG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AAATGCGGTA 360

GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG 420

ACAAAGTTTG ATTTTCCTGG AAGAGGAAAT AACCATTCCA GCTTTAAGTG GCGCTGGTAT 480

CATTTTGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAAT ATATAAATTC 540

AGGGGAACAG GCAAGC GGACTGGGAA GTCGATACAG AGAATGGCAA CTATGACTAT 600

CTTATGTATG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAAACTGG 660

GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTTA GAATAGATGC AGTGAAACAT 720

ATAAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA 780

ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAAA CTATTTGAAT 840

AAAACAAGTT GGAATCACTC GGTGTTTGAT GTTCCTCTCC ACTATAATTT GTACAATGCA 900

TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTTTAA ATGGTTCTGT GGTGCAAAAA 960

CATCCAACAC ATGCCGTTAC TTTTGTTGAT AACCATGATT CTCAGCCCGG GGAAGCATTG 1020

GAATCCTTTG TTCAACAATG GTTTAAACCA CTTGCATATG CATTGGTTCT GACAAGGGAA 1080

CAAGGTTATC CTTCCGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTTCCG 1140

GCTATGAAAT CTAAAATAGA CCCTCTTCTG CAGGCACGTC AAACTTTTGC CTATGGTACG 1200

CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC 1260

CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG 1320

TATGTGGGGA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC 1380

ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTTCTCTG TTAATGGAGG GTCCGTTTCG 1440

GTTTGGGTGA AGCAA 1455

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1455 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CATCATAATG GGACAAATGG GACGATGATG CAATACTTTG AATGGCACTT GCCTAATGAT 60

GGGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC 120

GCTATTTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAAA ATGATGTGGG GTATGGAGCC 180

TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTTCGTAC TAAGTATGGG 240

ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTTAT 300

GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC 360

GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGGG ACTACACAAT TGAGGCTTGG 420

ACTAAGTTTG ATTTTCCAGG GAGGGGTAAT ACATACTCAG ACTTTAAATG GCGTTGGTAT 480

CATTTCGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC 540

CGAGGTGATG GTAAGGCATG GGATTGGGAA GTAGATTCGG AAAATGGAAA TTATGATTAT 600

TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG 660

GGAGAATGGT ATACAAATAC ATTAAATCTT GATGGATTTA GGATCGATGC GGTGAAGCAT 720

ATTAAATATA GCTTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAAGAA 780

ATGTTTGCTG TTGCTGAATT TTGGAAAAAT GATTTAGGTG CCTTGGAGAA CTATTTAAAT 840

AAAACAAACT GGAATCATTC TGTCTTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG 900

TCAAATAGTG GAGGCAACTA TGACATGGCA AAACTTCTTA ATGGAACGGT TGTTCAAAAG 960

CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCACGATT CTCAACCTGG GGAATCATTA 1020

GAATCATTTG TACAAGAATG GTTTAAGCCA CTTGCTTATG CGCTTATTTT AACAAGAGAA 1080

CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCCAACACA TAGTGTCCCA 1140

GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA 1200

CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG 1260

CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG 1320

TACGTAGGGC AAAATAAAGC AGGTCAAGTT TGGCATGACA TAACTGGAAA TAAACCAGGA 1380

ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC 1440

ATTTGGGTGA AACGA 1455

(2) INFORMATION FOR SEQ ID NO: 11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1548 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCCGCACCGT TTAACGGCAC CATGATGCAG TATTTTGAAT GGTACTTGCC GGATGATGGC 60

ACGTTATGGA CCAAAGTGGC CAATGAAGCC AACAACTTAT CCAGCCTTGG CATCACCGCT 120

CTTTGGCTGC CGCCCGCTTA CAAAGGAACA AGCCGCAGCG ACGTAGGGTA CGGAGTATAC 180

GACTTGTATG ACCTCGGCGA ATTCAATCAA AAAGGGACCG TCCGCACAAA ATACGGAACA 240

AAAGCTCAAT ATCTTCAAGC CATTCAAGCC GCCCACGCCG CTGGAATGCA AGTGTACGCC 300

GATGTCGTGT TCGACCATAA AGGCGGCGCT GACGGCACGG AATGGGTGGA CGCCGTCGAA 360

GTCAATCCGT CCGACCGCAA CCAAGAAATC TCGGGCACCT ATCAAATCCA AGCATGGACG 420

AAATTTGATT TTCCCGGGCG GGGCAACACC TACTCCAGCT TTAAGTGGCG CTGGTACCAT 480

TTTGACGGCG TTGATTGGGA CGAAAGCCGA AAATTGAGCC GCATTTACAA ATTCCGCGGC 540

ATCGGCAAAG CGTGGGATTG GGAAGTAGAC ACGGAAAACG GAAACTATGA CTACTTAATG 600

TATGCCGACC TTGATATGGA TCATCCCGAA GTCGTGACCG AGCTGAAAAA CTGGGGGAAA 660

TGGTATGTCA ACACAACGAA CATTGATGGG TTCCGGCTTG ATGCCGTCAA GCATATTAAG 720

TTCAGTTTTT TTCCTGATTG GTTGTCGTAT GTGCGTTCTC AGACTGGCAA GCCGCTATTT 780

ACCGTCGGGG AATATTGGAG CTATGACATC AACAAGTTGC ACAATTACAT TACGAAAACA 840

GACGGAACGA TGTCTTTGTT TGATGCCCCG TTACACAACA AATTTTATAC CGCTTCCAAA 900

TCAGGGGGCG CATTTGATAT GCGCACGTTA ATGACCAATA CTCTCATGAA AGATCAACCG 960

ACATTGGCCG TCACCTTCGT TGATAATCAT GACACCGAAC CCGGCCAAGC GCTGCAGTCA 1020

TGGGTCGACC CATGGTTCAA ACCGTTGGCT TACGCCTTTA TTCTAACTCG GCAGGAAGGA 1080

TACCCGTGCG TCTTTTATGG TGACTATTAT GGCATTCCAC AATATAACAT TCCTTCGCTG 1140

AAAAGCAAAA TCGATCCGCT CCTCATCGCG CGCAGGGATT ATGCTTACGG AACGCAACAT 1200

GATTATCTTG ATCACTCCGA CATCATCGGG TGGACAAGGG AAGGGGGCAC TGAAAAACCA 1260

GGATCCGGAC TGGCCGCACT GATCACCGAT GGGCCGGGAG GAAGCAAATG GATGTACGTT 1320

GGCAAACAAC ACGCTGGAAA AGTGTTCTAT GACCTTACCG GCAACCGGAG TGACACCGTC 1380

ACCATCAACA GTGATGGATG GGGGGAATTC AAAGTCAATG GCGGTTCGGT TTCGGTTTGG 1440

GTTCCTAGAA AAACGACCGT TTCTACCATC GCTCGGCCGA TCACAACCCG ACCGTGGACT 1500

GGTGAATTCG TCCGTTGGAC CGAACCACGG TTGGTGGCAT GGCCTTGA 1548

(2) INFORMATION FOR SEQ ID NO: 12:
  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 1920 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: DNA (genomic)
  (ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION:421..1872
  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CGGAAGATTG GAAGTACAAA AATAAGCAAA AGATTGTCAA TCATGTCATG AGCCATGCGG 60

GAGACGGAAA AATCGTCTTA ATGCACGATA TTTATGCAAC GTTCGCAGAT GCTGCTGAAG 120

AGATTATTAA AAAGCTGAAA GCAAAAGGCT ATCAATTGGT AACTGTATCT CAGCTTGAAG 180

AAGTGAAGAA GCAGAGAGGC TATTGAATAA ATGAGTAGAA GCGCCATATC GGCGCTTTTC 240

TTTTGGAAGA AAATATAGGG AAAATGGTAC TTGTTAAAAA TTCGGAATAT TTATACAACA 300

TCATATGTTT CACATTGAAA GGGGAGGAGA ATCATGAAAC AACAAAAACG GCTTTACGCC 360

CGATTGCTGA CGCTGTTATT TGCGCTCATC TTCTTGCTGC CTCATTCTGC AGCAGCGGCG 420

GCA AAT CTT AAT GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC ATG CCC 468

AAT GAC GGC CAA CAT TGG AGG CGT TTG CAA AAC GAC TCG GCA TAT TTG 516

GCT GAA CAC GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT AAG GGA 564

ACG AGC CAA GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT GAT TTA 612

GGG GAG TTT CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC ACA AAA 660

GGA GAG CTG CAA TCT GCG ATC AAA AGT CTT CAT TCC CGC GAC ATT AAC 708

GTT TAC GGG GAT GTG GTC ATC AAC CAC AAA GGC GGC GCT GAT GCG ACC 756

GAA GAT GTA ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC CGC GTA 804

ATT TCA GGA GAA CAC CTA ATT AAA GCC TGG ACA CAT TTT CAT TTT CCG 852

GGG CGC GGC AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC CAT TTT 900

GAC GGA ACC GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC TAT AAG 948

TTT CAA GGA AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC GGC AAC 996

TAT GAT TAT TTG ATG TAT GCC GAC ATC GAT TAT GAC CAT CCT GAT GTC 1044

GCA GCA GAA ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA CTG CAA 1092

TTG GAC GGT TTC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT TCT TTT 1140

TTG CGG GAT TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG GAA ATG 1188

TTT ACG GTA GCT GAA TAT TGG CAG AAT GAC TTG GGC GCG CTG GAA AAC 1236

TAT TTG AAC AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG CCG CTT 1284

CAT TAT CAG TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT GAT ATG 1332

AGG AAA TTG CTG AAC GGT ACG GTC GTT TCC AAG CAT CCG TTG AAA TCG 1380

GTT ACA TTT GTC GAT AAC CAT GAT ACA CAG CCG GGG CAA TCG CTT GAG 1428

TCG ACT GTC CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT ATT CTC 1476

ACA AGG GAA TCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG TAC GGG 1524

ACG AAA GGA GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC AAA ATT 1572

GAA CCG ATC TTA AAA GCG AGA AAA CAG TAT GCG TAC GGA GCA CAG CAT 1620

GAT TAT TTC GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA GGC GAC 1668

AGC TCG GTT GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC GGA CCC 1716

GGT GGG GCA AAG CGA ATG TAT GTC GGC CGG CAA AAC GCC GGT GAG ACA 1764

TGG CAT GAC ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC AAT TCG 1812

GAA GGC TGG GGA GAG TTT CAC GTA AAC GGC GGG TCG GTT TCA ATT TAT 1860

GTT CAA AGA TAG AAGAGCAGAG AGGACGGATT TCCTGAAGGA AATCCGTTTT 1912

TTTATTTT 1920

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2084 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:343..1794

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCCCCGCACA TACGAAAAGA CTGGCTGAAA ACATTGAGCC TTTGATGACT GATGATTTGG 60

CTGAAGAAGT GGATCGATTG TTTGAGAAAA GAAGAAGACC ATAAAAATAC CTTGTCTGTC 120

ATCAGACAGG GTATTTTTTA TGCTGTCCAG ACTGTCCGCT GTGTAAAAAT AAGGAATAAA 180

GGGGGGTTGT TATTATTTTA CTGATATGTA AAATATAATT TGTATAAGAA AATGAGAGGG 240

AGAGGAAACA TGATTCAAAA ACGAAAGCGG ACAGTTTCGT TCAGACTTGT GCTTATGTGC 300

ACGCTGTTAT TTGTCAGTTT GCCGATTACA AAAACATCAG CC GTA AAT GGC ACG 354

CTG ATG CAG TAT TTT GAA TGG TAT ACG CCG AAC GAC GGC CAG CAT TGG 402

AAA CGA TTG CAG AAT GAT GCG GAA CAT TTA TCG GAT ATC GGA ATC ACT 450

GCC GTC TGG ATT CCT CCC GCA TAC AAA GGA TTG AGC CAA TCC GAT AAC 498

GGA TAC GGA CCT TAT GAT TTG TAT GAT TTA GGA GAA TTC CAG CAA AAA 546

GGG ACG GTC AGA ACG AAA TAC GGC ACA AAA TCA GAG CTT CAA GAT GCG 594

ATC GGC TCA CTG CAT TCC CGG AAC GTC CAA GTA TAC GGA GAT GTG GTT 642

TTG AAT CAT AAG GCT GGT GCT GAT GCA ACA GAA GAT GTA ACT GCC GTC 690

GAA GTC AAT CCG GCC AAT AGA AAT CAG GAA ACT TCG GAG GAA TAT CAA 738

ATC AAA GCG TGG ACG GAT TTT CGT TTT CCG GGC CGT GGA AAC ACG TAC 786

AGT GAT TTT AAA TGG CAT TGG TAT CAT TTC GAC GGA GCG GAC TGG GAT 834

GAA TCC CGG AAG ATC AGC CGC ATC TTT AAG TTT CGT GGG GAA GGA AAA 882

GCG TGG GAT TGG GAA GTA TCA AGT GAA AAC GGC AAC TAT GAC TAT TTA 930

ATG TAT GCT GAT GTT GAC TAC GAC CAC CCT GAT GTC GTG GCA GAG ACA 978

AAA AAA TGG GGT ATC TGG TAT GCG AAT GAA CTG TCA TTA GAC GGC TTC 1026

CGT ATT GAT GCC GCC AAA CAT ATT AAA TTT TCA TTT CTG CGT GAT TGG 1074

GTT CAG GCG GTC AGA CAG GCG ACG GGA AAA GAA ATG TTT ACG GTT GCG 1122

GAG TAT TGG CAG AAT AAT GCC GGG AAA CTC GAA AAC TAC TTG AAT AAA 1170

ACA AGC TTT AAT CAA TCC GTG TTT GAT GTT CCG CTT CAT TTC AAT TTA 1218

CAG GCG GCT TCC TCA CAA GGA GGC GGA TAT GAT ATG AGG CGT TTG CTG 1266

GAC GGT ACC GTT GTG TCC AGG CAT CCG GAA AAG GCG GTT ACA TTT GTT 1314

GAA AAT CAT GAC ACA CAG CCG GGA CAG TCA TTG GAA TCG ACA GTC CAA 1362

ACT TGG TTT AAA CCG CTT GCA TAC GCC TTT ATT TTG ACA AGA GAA TCC 1410

GGT TAT CCT CAG GTG TTC TAT GGG GAT ATG TAC GGG ACA AAA GGG ACA 1458

TCG CCA AAG GAA ATT CCC TCA CTG AAA GAT AAT ATA GAG CCG ATT TTA 1506

AAA GCG CGT AAG GAG TAC GCA TAC GGG CCC CAG CAC GAT TAT ATT GAC 1554

CAC CCG GAT GTG ATC GGA TGG ACG AGG GAA GGT GAC AGC TCC GCC GCC 1602

AAA TCA GGT TTG GCC GCT TTA ATC ACG GAC GGA CCC GGC GGA TCA AAG 1650

CGG ATG TAT GCC GGC CTG AAA AAT GCC GGC GAG ACA TGG TAT GAC ATA 1698

ACG GGC AAC CGT TCA GAT ACT GTA AAA ATC GGA TCT GAC GGC TGG GGA 1746

GAG TTT CAT GTA AAC GAT GGG TCC GTC TCC ATT TAT GTT CAG AAA TAA 1794

GGTAATAAAA AAACACCTCC AAGCTGAGTG CGGGTATCAG CTTGGAGGTG CGTTTATTTT 1854

TTCAGCCGTA TGACAAGGTC GGCATCAGGT GTGACAAATA CGGTATGCTG GCTGTCATAG 1914

GTGACAAATC CGGGTTTTGC GCCGTTTGGC TTTTTCACAT GTCTGATTTT TGTATAATCA 1974

ACAGGCACGG AGCCGGAATC TTTCGCCTTG GAAAAATAAG CGGCGATCGT AGCTGCTTCC 2034

AATATGGATT GTTCATCGGG ATCGCTGCTT TTAATCACAA CGTGGGATCC 2084

(2) INFORMATION FOR SEQ ID NO: 13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1455 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CATCATAATG GAACAAATGG TACTATGATG CAATATTTCG AATGGTATTT GCCAAATGAC 60

GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA 120

GCTGTATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGTAGG TTATGGAGCC 180

TATGATTTAT ATGATCTTGG AGAGTTTAAC CAGAAGGGGA CGGTTCGTAC AAAATATGGA 240

ACACGCAACC AGCTACAGGC TGCGGTGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT 300

GGTGATGTCG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AAATGCGGTA 360

GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG 420

ACAAAGTTTG ATTTTCCTGG AAGAGGAAAT AACCATTCCA GCTTTAAGTG GCGCTGGTAT 480

CATTTTGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAAT ATATAAATTC 540

AGGGGAACAG GCAAGGCCTG GGACTGGGAA GTCGATACAG AGAATGGCAA CTATGACTAT 600

CTTATGTATG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAAACTGG 660

GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTTA GAATAGATGC AGTGAAACAT 720

ATAAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA 780

ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAAA CTATTTGAAT 840

AAAACAAGTT GGAATCACTC GGTGTTTGAT GTTCCTCTCC ACTATAATTT GTACAATGCA 900

TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTTTAA ATGGTTCTGT GGTGCAAAAA 960

CATCCAACAC ATGCCGTTAC TTTTGTTGAT AACCATGATT CTCAGCCCGG GGAAGCATTG 1020

GAATCCTTTG TTCAACAATG GTTTAAACCA CTTGCATATG CATTGGTTCT GACAAGGGAA 1080

CAAGGTTATC CTTCCGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTTCCG 1140

GCTATGAAAT CTAAAATAGA CCCTCTTCTG CAGGCACGTC AAACTTTTGC CTATGGTACG 1200

CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC 1260

CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG 1320

TATGTGGGGA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC 1380

ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTTCTCTG TTAATGGAGG GTCCGTTTCG 1440

GTTTGGGTGA AGCAA 1455

(2) INFORMATION FOR SEQ ID NO: 14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1455 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CATCATAATG GGACAAATGG GACGATGATG CAATACTTTG AATGGCACTT GCCTAATGAT 60

GGGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC 120

GCTATTTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAAA ATGATGTGGG GTATGGAGCC 180

TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTTCGTAC TAAGTATGGG 240

ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTTAT 300

GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC 360

GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGGG ACTACACAAT TGAGGCTTGG 420

ACTAAGTTTG ATTTTCCAGG GAGGGGTAAT ACATACTCAG ACTTTAAATG GCGTTGGTAT 480

CATTTCGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC 540

CGAGGTGATG GTAAGGCATG GGATTGGGAA GTAGATTCGG AAAATGGAAA TTATGATTAT 600

TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG 660

GGAGAATGGT ATACAAATAC ATTAAATCTT GATGGATTTA GGATCGATGC GGTGAAGCAT 720

ATTAAATATA GCTTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAAGAA 780

ATGTTTGCTG TTGCTGAATT TTGGAAAAAT GATTTAGGTG CCTTGGAGAA CTATTTAAAT 840

AAAACAAACT GGAATCATTC TGTCTTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG 900

TCAAATAGTG GAGGCAACTA TGACATGGCA AAACTTCTTA ATGGAACGGT TGTTCAAAAG 960

CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCACGATT CTCAACCTGG GGAATCATTA 1020

GAATCATTTG TACAAGAATG GTTTAAGCCA CTTGCTTATG CGCTTATTTT AACAAGAGAA 1080

CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCCAACACA TAGTGTCCCA 1140

GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA 1200

CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG 1260

CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG 1320

TACGTAGGGC AAAATAAAGC AGGTCAAGTT TGGCATGACA TAACTGGAAA TAAACCAGGA 1380

ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC 1440

ATTTGGGTGA AACGA 1455

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 60 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Forward Primer FSA"
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) LOCATION: 22-27,29,31-33,41
(D): OTHER INFORMATION: /Note= 1: 35% A, 65% C
2: 83% G, 17% A
3: 63% G, 37% T
4: 86% G, 14% A
5: 85% G, 15% C
6: 50% T, 50% C
7: 95% A, 5%G
8: 58% G, 37% A, 5% T
9: 86% C, 13% A, 1% G
10: 83% T, 17% G
11: 92% G, 8% C
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

caaaatcgta tctacaaatt c123456a7g 8910tgggatt
11ggaagtaga ttcggaaaat
60

(2) INFORMATION FOR SEQ ID NO: 16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Reverse Primer RSA"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

gaatttgtag atacgatttt g
21

(2) INFORMATION FOR SEQ ID NO: 17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer B1"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CGATTGCTGA CGCTGTTATT TGCG 24

(2) INFORMATION FOR SEQ ID NO: 18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer Y2"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CTTGTTCCCT TGTCAGAACC AATG 24

(2) INFORMATION FOR SEQ ID NO: 19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 101458"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GTCATAGTTG CCGAAATCTG TATCGACTTC 30

(2) INFORMATION FOR SEQ ID NO: 20:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 101638"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CCCAGTCCCA CGTACGTCCC CTGAATTTATATA TTTTG 35

(2) INFORMATION FOR SEQ ID NO: 21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Oligo 1"
(A) NAME/KEY: misc-feature
(B) LOCATION: 12
(D): OTHER INFORMATION: /Note=N= 25% A, 25% C, 25% G, 25% T.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CCCAGTCCCA GNTCTTTCCC CTGAATTTAT ATATTTTG 38

(2) INFORMATION FOR SEQ ID NO: 22:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer X2"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GCGTGGACAA AGTTTGATTT TCCTG 25

(2) INFORMATION FOR SEQ ID NO: 23:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA01"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

2) INFORMATION FOR SEQ ID NO: 24:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA03"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:
GCATTGGATG CTTTTGAACA ACCG 24

2) INFORMATION FOR SEQ ID NO: 25:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA07"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CGCAAAATGA TATCGGGTAT GGAGCC 26

(2) INFORMATION FOR SEQ ID NO: 26:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA20"
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) LOCATION: 13,14
(D): OTHER INFORMATION: /Note:S= mixture of C and G
W= mixture of A and T
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GTGATGAACC ACSWAGGTGG AGCTGATGC 29

(2) INFORMATION FOR SEQ ID NO: 27:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA14"
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) LOCATION: 13,14
(D): OTHER INFORMATION: /Note:R= mixture of A and G
Y= mixture of C and T
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GATGGTGTAT GGRYCAATCA CGACAATTCC 30

2) INFORMATION FOR SEQ ID NO: 28:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA15"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:
GGTGTATGGG ATAACTCACG ACAATTCC 28

2) INFORMATION FOR SEQ ID NO: 29:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA16"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:
GGTGTATGGG ATCTCTCACG ACAATTCC 28

2) INFORMATION FOR SEQ ID NO: 30:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA17"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:
GGGATCAATC ACGAAATTTC CAAAATCGTA TC 32

2) INFORMATION FOR SEQ ID NO: 31:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA18"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:
GGGATCAATC ACGACTCTTC CAAAATCGTA TC 32

2) INFORMATION FOR SEQ ID NO: 32:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA06"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:
GGAAATTATG ATTATATCAT GTATGCAGAT GTAG 34

2) INFORMATION FOR SEQ ID NO: 33:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA09"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:
GCTGAATTTT GGTCGAATGA TTTAGGTGCC 30

2) INFORMATION FOR SEQ ID NO: 34:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA11"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:
GCTGAATTTT GGTCGAATGA TTTAGGTGCC 30

2) INFORMATION FOR SEQ ID NO: 35:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA21"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:
GAATTTTGGA AGTACGATTT AGGTCGG 27

(2) INFORMATION FOR SEQ ID NO: 36:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA12"
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) LOCATION: 12,13
(D): OTHER INFORMATION: /Note:R= mixture of A and G
Y= mixture of C and T
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:
GGAAAAACGA TRYCGGTGCC TTGGAGAAC 29

(2) INFORMATION FOR SEQ ID NO: 37:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA13"

(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) LOCATION: 14,15
(D): OTHER INFORMATION: /Note:R= mixture of A and G
Y= mixture of C and T

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37

GATTTAGGTG CCTRYCAGAA CTATTTA 27

2) INFORMATION FOR SEQ ID NO: 38:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA08"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:
CCCCCTTCAT GAGAATCTTT ATAACG 26

2) INFORMATION FOR SEQ ID NO: 39:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA04"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:
GAATCCGAAC CTCATTACAC ATTCG 25

2) INFORMATION FOR SEQ ID NO: 40:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA05"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:
CGGATGGACT CGAGAAGGAA ATACCACG 38

2) INFORMATION FOR SEQ ID NO: 41:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:

(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA10"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:
CGTAGGGCAA AATCAGGCCG GTCAAGTTTG G 31

2) INFORMATION FOR SEQ ID NO: 42:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA22"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:
CATAACTGGA AATCGCCCGG GAACAGTTAC G 31

(2) INFORMATION FOR SEQ ID NO: 43:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/ KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA19"
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) LOCATION: 12
(D): OTHER INFORMATION: /Note:W= mixture of A and T
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43
CTGGAAATAA AWCCGGAACA GTTACG 36

2) INFORMATION FOR SEQ ID NO: 44:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA23"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:
GGAAATAAAC CAGGACCCGT TACGATCAAT GC 32

2) INFORMATION FOR SEQ ID NO: 45:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:

(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA32"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:
GAGGCTTGGA CTAGGTTTGA TTTTCCAG 28

2) INFORMATION FOR SEQ ID NO: 46:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer DA31"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:
GCTGAATTTT GGCGCAATGA TTTAGGTGCC 30

2) INFORMATION FOR SEQ ID NO: 47:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer bm4"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:
GTGTTTGACG TCCCGCTTCA TGAGAATTTA CAGG 34

2) INFORMATION FOR SEQ ID NO: 48:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer bm5"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:
GTGTTTGACG TCCCGCTTCA TAAGAATTTA CAGG 34

2) INFORMATION FOR SEQ ID NO: 49:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer bm6"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:
GTGTTTGACG TCCCGCTTCA TGCCAATTTA CAGG 34

2) INFORMATION FOR SEQ ID NO: 50:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer bm8"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:
AGGGAATCCG GATACCCTGA GGTTTTCTAC GG 32

2) INFORMATION FOR SEQ ID NO: 51:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer bm11"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:
GATGTGGTTT TGGATCATAA GGCCGGCGCT GATG 34

2) INFORMATION FOR SEQ ID NO: 52
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer p1"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:
CTGTTATTAA TGCCGCCAAA CC 22

2) INFORMATION FOR SEQ ID NO: 53:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer p2"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

GGAAAAGAAA TGTTTACGGT TGCG 24

2) INFORMATION FOR SEQ ID NO: 54:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer p3"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:
GAAATGAAGC GGAACATCAA ACACG 25

2) INFORMATION FOR SEQ ID NO: 55:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer p4"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:
GTATGATTTA GGAGAATTCC 20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00471

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/28, C11D 3/386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, PAJ, BIOSIS, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9741213 A1 (NOVO NORDISK A/S), 6 November 1997 (06.11.97)	1-39
	--	
X	WO 9623874 A1 (NOVO NORDISK A/S), 8 August 1996 (08.08.96), See abstract, page 34 and claim 48	1-39
	--	
X	WO 9623873 A1 (NOVO NORDISK A/S), 8 August 1996 (08.08.96), See page 6, line 9-15, ex 4 and 5, page 75-77	1-39
	--	
X	WO 9100353 A2 (GIST-BROCADES N.V.), 10 January 1991 (10.01.91), page 4, line 16, claim 3	1-39
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 February 1999	16 -02- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00471

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9535382 A1 (NOVO NORDISK B.V.), 28 December 1995 (28.12.95), See abstract and claims --	1-39
A	WO 9510603 A1 (NOVO NORDISK A/S), 20 April 1995 (20.04.95) -- --------	1-39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00471

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The claimed inventions relate to variants of a parent Termamyl-like alpha-amylase.
A large number of mutations or combinations of mutations are suggested, which give
a)improved pH stability at a pH from 8 to 10.5 and/or
b) improved Ca2+ stability at pH 8 to 10.5 and/or
c) increased specific activity at temperatures from 10 to 60C.
Mutations of Termamyl-like alpha-amylases are well-known in the art , see e.g. WO 96/23874.
No common theory for all the mutations are suggested in the present application. Therefore no "special technical feature" that makes a contribution to the prior art, as demanded in PCT rule 13.2, has been found.
The application claims a large number of inventions, in spite of this all inventions have been searched.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

21/12/98

International application No.
PCT/DK 98/00471

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9741213 A1	06/11/97	AU 2692897 A	19/11/97
WO 9623874 A1	08/08/96	AU 4483496 A	21/08/96
		BR 9607013 A	28/10/97
		CA 2211316 A	08/08/96
		CN 1172501 A	04/02/98
		EP 0808363 A	26/11/97
WO 9623873 A1	08/08/96	AU 4483396 A	21/08/96
		BR 9607735 A	14/07/98
		CA 2211405 A	08/08/96
		CN 1172500 A	04/02/98
		EP 0815208 A	07/01/98
WO 9100353 A2	10/01/91	AT 166922 T	15/06/98
		AU 638263 B	24/06/93
		AU 5953890 A	17/01/91
		BG 61081 B	31/10/96
		CA 2030554 A	30/12/90
		CN 1050220 A	27/03/91
		DE 69032360 D,T	03/12/98
		EP 0410498 A,B	30/01/91
		SE 0410498 T3	
		ES 2117625 T	16/08/98
		FI 910907 D	00/00/00
		JP 4500756 T	13/02/92
		PT 94560 A,B	08/02/91
		US 5364782 A	15/11/94
WO 9535382 A2	28/12/95	AU 685638 B	22/01/98
		AU 2524795 A	15/01/96
		EP 0772684 A	14/05/97
WO 9510603 A1	20/04/95	AU 7807494 A	04/05/95
		BR 9407767 A	18/03/97
		CA 2173329 A	20/04/95
		CN 1134725 A	30/10/96
		EP 0722490 A	24/07/96
		FI 961524 A	30/05/96
		JP 9503916 T	22/04/97
		US 5753460 A	19/05/98
		US 5801043 A	01/09/98